Estudio polifásico del patógeno de peces *Yersinia ruckeri*

Asmine V. Bastardo Espinoza

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Departamento de Microbiología y Parasitología
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ESTUDIO POLIFÁSICO DEL PATÓGENO DE PECES *Yersinia ruckeri*

Memoria que presenta
Asmine V. Bastardo Espinoza
para optar al grado de
Doctora en Biología

Fdo: Asmine V. Bastardo Espinoza
Santiago de Compostela,
Octubre 2012

Jesús López Romalde, Catedrático del Departamento de Microbiología y Parasitología de la Universidad de Santiago de Compostela y **Carmen Urquía Ravelo Vívenes**, Investigadora de la Fundación La Salle de Ciencias Naturales

Informan: que la presente Tesis Doctoral titulada: "**Estudio polifásico del patógeno de peces *Yersinia ruckeri*",** que presenta **Asmine Victoria Bastardo Espinoza** para optar al grado de Doctora en Biología, ha sido realizada en el Departamento de Microbiología y Parasitología bajo mi dirección, y considerando que se haya concluída, autorizo su presentación para que pueda ser juzgada por el tribunal correspondiente.

En Santiago de Compostela, a 22 de septiembre de 2012

Dr. Jesús L. Romalde **Dra. Carmen Ravelo**

Este trabajo se desarrolló en el marco de dos proyectos de investigación, AGL2006-13208 y AGL2010-18438 subvencionados por el Ministerio de Ciencia e Innovación dentro del Subprograma de proyectos de investigación fundamental no orientada.

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Asmine

INDICE

Abreviaturas y siglas

BT1: Biotipo 1

BT2: Biotipo 2

CC: Complejo clonal (*Clonal complex*)

CHEF: Electroforesis en campo eléctrico homogéneo en contorno cerrado (*Contour-clamped homogeneous electric field*)

CMI: Concentración mínima inhibitoria

DGGE: Electroforesis en gel de gradiente desnaturalizante (*Denaturing Gradient Gel Electrophoresis*)

DL_{50}: Dosis letal media

DNA: Acido desoxirribonucleico (*Deoxyribonucleic acid*)

ECP: Productos extracelulares (*Extracellular products*)

ELISA: Ensayo por inmunoabsorción ligado a enzimas (*Enzyme-Linked ImmunoSorbent Assay*)

ERIC-PCR: Reacción en cadena de la polimerasa de secuencias consenso intergénicas repetitivas (*Enterobacterial repetitive intergenic consensus*)

ERM: enfermedad de la boca roja (*Enteric redmouth disease*)

FAO: Organización Mundial para la Alimentación y la Agricultura (*Food and Agriculture Organization of the United Nations*)

FIGE: Electroforesis en gel de campo invertido (*Field-inversion gel electrophoresis*)

G+C. Contenido guanina + citosina

GFP: Proteína fluorescente verde

HK: Genes esenciales (*Housekeeping genes*)

HMP: Proteína termo-modificable (*Heat modifiable protein*)

HSF: Factor sensible a la temperatura (*Heat Sensible Factor*)

IFAT: Ensayo de anticuerpos por inmunofluorescencia directa (*Indirect Immunofluorescent Antibody Test*)

LAMP: Amplificación isotérmica en bucle del DNA (*Loop-mediated isothermal amplification*)

LPS: Lipopolisacáridos (*Lipopolysaccharide*)

MDa: Mega Daltons

MLEE: Análisis electroforético de múltiples locis enzimáticos

MLST: Tipado de secuencias multilócicas (*Multilocus sequence typing*)

OMP: Proteínas de membrana externa (*Outer membrane protein*)

PAP: Proteína asociada a peptidoglicano (*Peptidoglycan-associated protein*)

PFGE: Electroforésis en campo pulsado (*Pulsed field gel electrophoresis*)

qPCR: Reacción en cadena de la polimerasa cuantitativa

REP-PCR: Reacción en cadena de la polimerasa de elementos palindrómicos extragénicos repetitivos (*Enterobacterial repetitive extragenic palindromic*)

RFLP: análisis de polimorfismos en la longitud de fragmentos de restricción (*Restriction fragment length polymorphism)*

SDS-PAGE electroforesis en geles de poliacrilamida (*Sodium-dodecyl sulphate polyacrylamide gel electrophoresis*)

ST: Tipo de secuencia (*Sequence type*)

T3SS: Sistema de secreción tipo III (*Type three secretion system*)

T4SS: Sistema de secreción tipo IV (*Type four secretion system*)

T5SS: Sistema de secreción tipo V (*Type five secretion system*)

TSA. Agar tripticasa de soja (*Trypticase soy agar*)

UFC: Unidades formadoras de colonia

USA: Estados Unidos de América

VCM: Volumen corpuscular medio

VP: Reacción Voges-Proskauer

RESUMEN

Resumen

La enfermedad de la boca roja (ERM), una de las patologías más importantes que afecta a peces salmónidos, está causada por la bacteria *Yersinia ruckeri*. Este patógeno constituyó un importante factor limitante en el cultivo de salmónidos en el mundo. El pronto desarrollo de una vacuna comercial permitió el control de la enfermedad y el desarrollo de los cultivos de este grupo de peces. Sin embargo, en los últimos años se están detectando brotes de ERM en peces previamente vacunados, causados por cepas de *Y. ruckeri* inmóviles (biotipo 2) y/o diferentes del serotipo típico O1a móvil (biotipo 1). Este hecho indica que se ha producido un cambio antigénico en las cepas con respecto a las de hace algunos años. Esta tesis se centró en el estudio de la diversidad intraespecífica y evolución molecular de *Y. ruckeri*, para dilucidar la diseminación, diversificación y mantenimiento de este importante patógeno, así como también las interrelaciones filogenéticas entre estos aislados y sus aspectos poblacionales.

El estudio de diferentes cepas de *Y. ruckeri* aisladas recientemente de brotes de ERM en peces vacunados, reveló una gran variabilidad a nivel de sus características bioquímicas, serológicas y genéticas. Los resultados obtenidos evidenciaron que el biotipo 2 de *Y. ruckeri* puede también estar asociado con el grupo serológico O1b. Además, se describieron por primera vez cepas de *Y. ruckeri* pertenecientes al biotipo 2 aisladas de brotes de ERM en Portugal y Suramérica. También se desarrolló un esquema de tipado de secuencias multilócicas (MLST) para *Y. ruckeri* en base a la secuencias de fragmentos internos de seis genes esenciales. Este esquema MLST se aplicó a 103 cepas de *Y. ruckeri* de diferentes fenotipos, aisladas de diferente huéspedes en diversas áreas geográficas, así como de origen ambiental. Las secuencias obtenidas a partir de

este trabajo se depositaron en una base de datos pública (http://publmst.org/yruckeri/) y son de libre acceso.

Los estudios poblacionales indicaron que *Y. ruckeri* ha experimentado cambios en la población, inducidos por fuerzas biogeográficas en el pasado y, más recientemente, por procesos de adaptación forzada por la expansión de la acuicultura. Por otra parte, los estudios evolutivos permitieron determinar una tasa evolutiva promedio en *Y. ruckeri* de 2.5 x 10^{-5} sustituciones por nucleótido/sitio/año, sugiriendo que este patógeno puede evolucionar más rápido de lo comúnmente observado en otras bacterias. Además, la fuerte estructuración de diferentes linajes de *Y. ruckeri* en diferentes áreas geográficas observada en este estudio, sugiere que el mantenimiento enzoótico (evolución *in situ)* de *Y. ruckeri* puede ser otra vía alternativa, importante en el mantenimiento de la ERM en el mundo propiciada también por la acuicultura. Desde este punto de vista, podemos teorizar que la rápida diversificación de *Y. ruckeri*, así como la emergencia y aumento de los nuevos casos de ERM en peces vacunados en todo el mundo, es una consecuencia de diferentes factores relacionados con la acuicultura intensiva.

SUMMARY

Summary

The enteric redmouth disease (ERM) is one of the most important diseases affecting salmonids, caused by the bacterium *Yersinia ruckeri*. This pathogen was an important limiting factor in salmonid culture in the world. The early development of a commercial vaccine allowed control of the disease and the development of the culture of these fish species. However, in recent years ERM outbreaks in previously vaccinated fish are being detected, caused by non-motile strains of *Y. ruckeri* (biotype 2) and/or by strains belonging different serotypes to typical motile (biotype 1) O1a strain. This fact indicates that antigenic changes have occurred in strains respect to a few years ago. This thesis focused on the study of intraspecific diversity and molecular evolution of *Y. ruckeri*, in order to elucidate the spread, diversification and maintenance of this important pathogen, as well as the phylogenetic relationships among these isolates and also population aspects.

The study of several strains of *Y. ruckeri* recently isolated in ERM outbreaks from vaccinated fish, revealed a great variability in the biochemical, serological and genetic characteristics. The results showed that the biotype 2 of *Y. ruckeri* may also be associated with O1b serogroup. Further we described the first strains of *Y. ruckeri* belonging to biotype 2 isolated from outbreaks of ERM in Portugal and South America. A multilocus sequence typing (MLST) scheme was also developed for *Y. ruckeri* based on the sequence of internal fragments of six essential genes. This MLST scheme was applied to 103 *Y. ruckeri* strains of different phenotypes isolated from several geographical areas and environmental origin. The sequences obtained from this study were hosted in a public database (http://publmst.org/yruckeri/) and are freely available.

Population studies indicated that *Y. ruckeri* has undergone population changes induced by biogeographic forces in the past and more recently by adaptation processes forced by the expansion of aquaculture. Moreover, evolutionary studies allowed to determine average evolutionary rate in *Y. ruckeri* of 2.5 x 10^{-5} nucleotide substitutions/site/year, suggesting that this pathogen can evolve faster than commonly observed in other bacteria. Furthermore, the strong structuring of different lineages of *Y. ruckeri* in different geographic areas observed in this study, suggested that enzootic maintenance (evolution *in situ*) of *Y. ruckeri,* also favored by aquaculture, may be another alternative way for perpetuation of ERM in the world. From this point of view, we theorize that the rapid diversification and spread of *Y. ruckeri,* as well as the emergence and increase of new cases of ERM in vaccinated fish worldwide, is a result of different factors related to intensive aquaculture.

1. INTRODUCCIÓN GENERAL

Modificado parcialmente de:

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1. INTRODUCCIÓN GENERAL

En la actualidad, la acuicultura es uno de los sectores productores de alimentos en más rápido crecimiento, y representa más del 47% de la oferta de pescado en el mundo (Tacón y col., 2010). La Organización Mundial para la Alimentación y la Agricultura (FAO) estima que hacia 2030 la acuicultura producirá el 65% de los alimentos de procedencia acuática destinados al consumo humano.

Dentro de la producción acuícola de peces, el cultivo de salmónidos es el más practicado a nivel mundial (Fig. 1.1), presentando un mayor grado de desarrollo técnico y una explotación más racional. Así, la trucha arcoíris (*Oncorhynchus mykiss*) y el salmón del atlántico (*Salmo salar*) representan por sí mismos un 40% de la producción total. A nivel mundial, la producción de salmónidos fue en 2007 de aproximadamente 2 millones de toneladas, con un valor global de 1.100 millones de dólares, contribuyendo la trucha arcoíris con 600.000 toneladas a esta cifra (FAO, 2007).

En términos de producción, uno de los mayores problemas lo constituyen las enfermedades infecciosas, causantes de las mayores pérdidas económicas en la industria de la acuicultura, debido tanto a los costes de los tratamientos como a la muerte de los peces. Entre estas enfermedades infecciosas destacan de forma importante las de origen bacteriano. En la Tabla 1.1 se incluyen detalles de diferentes patógenos bacterianos en especies de peces cultivados.

Dado que la acuicultura continúa creciendo en todo el mundo, es de suma importancia la constante investigación de enfermedades bacterianas, con el fin de evitar pérdidas y mejorar las decisiones de gestión de la salud de los cultivos. El presente trabajo de investigación se centra en el estudio de la diversidad

intraespecífica y evolución molecular de *Y. ruckeri*, uno de los patógenos bacterianos más importantes para el cultivo de salmónidos a nivel mundial.

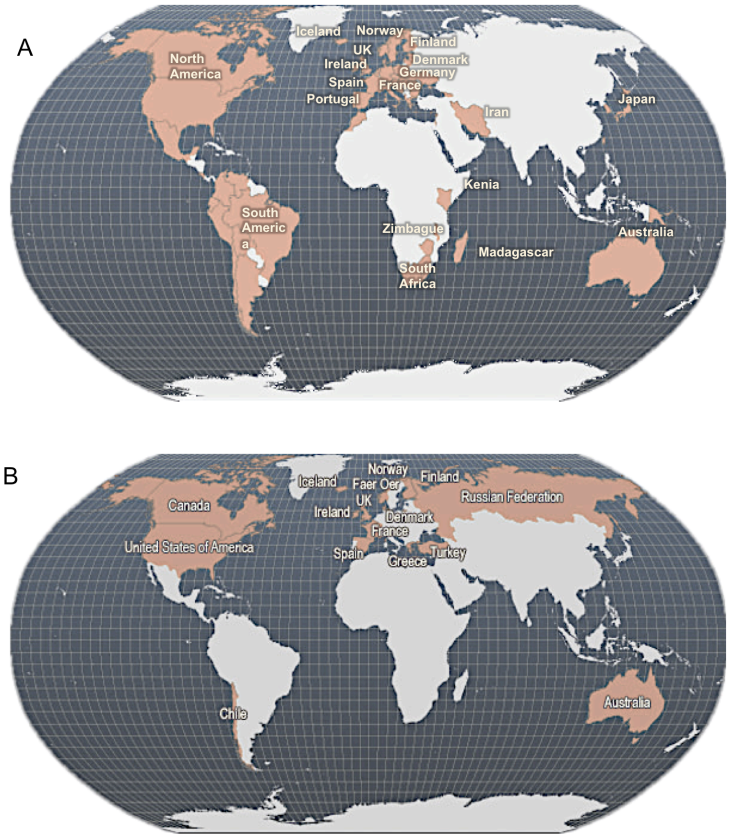

Figura 1.1. Principales países productores de *Oncorhynchus mykiss* (A) y *Salmo salar* (B) (FAO Estadística Pesquera, 2006).

Table 1.1 Principales patógenos bacterianos de peces (Adaptado de Roberts, 2001 y Austin y Austin, 2007)

PATÓGENO	ENFERMEDAD
Bacterias Gram-negativas con movilidad deslizante	
Flavobacteriaceae:	
Flavobacterium branchiophilum	Enfermedad bacteriana de las branquias
Flavobacterium columnare	Enfermedad de la columna (columnaris)
Flavobacterium psychrophilum	Enfermedad del agua fría
Tenacibaculum maritimum	Tenacibaculosis (flexibacteriosis)
Bacilos anaeróbicos facultativos Gram-negativos	
Enterobacteriaceae	
Edwardsiella tarda	Edwardsielosis
Edwardsiella ictaluri	Edwardsielosis del bagre
Yersinia ruckeri	Yersiniosis (enfermedad de la boca roja)
Vibrionaceae y Photobacteriaceae	
Vibrio anguillarum	Vibriosis
Vibrio ordalii	Vibriosis
Aliivibrio (Vibrio) salmonicida	Vibriosis de agua fría
Moritella (Vibrio) viscosa	Enfermedad ulcerosa de agua fría
Photobacterium damselae subsp. *piscicida*	Fotobacteriosis, Pasteurelosis o Pseudotuberculosis
Aeromonadaceae	
Aeromonas hydrophila	Septicemia
Aeromonas salmonicida	Forunculosis
Bacterias aerobicas Gram-positivos	
Renibacterium salmoninarum	Enfermedad bacteriana del riñón (BKD)
Carnobacterium piscicola	Lactobacilosis
Vagococcus salmoninarum	Lactobacilosis
Lactococcus piscium	Lactococosis
Lactococcus garvieae	Lactococosis
Streptococcus ineae	Estreptococosis
Streptococcus galactiae	Estreptococosis
Streptococcus disgalactiae	Estreptococosis
Streptococcus parauberis	Estreptococosis
Bacilos ácido-alcohol resistentes y filamentosos	
Mycobacterium marinum	Micobacteriosis
Mycobacterium fortuitum	
Mycobacterium chelonae	
Nocardiaceae	
Nocardia asteroides	Nocardiosis
Nocardia kampachi	
Rickettsias/Chlamydias: parásitos intracelulares obligados	
Piscirickettsia salmonis	Rickettsiosis
Epitheliocystis	Epiteliocistis
RLO (Rickettsia like organism)	Síndrome de la marca roja

1.1. La enfermedad entérica de la boca roja (ERM del inglés "Enteric Redmouth disease")

Yersinia ruckeri es el agente etiológico de la enfermedad entérica de la boca roja (ERM) o yersiniosis la cual produce importantes pérdidas económicas en la acuicultura de muchos países. El nombre enfermedad de la boca roja se empleó para distinguir la enfermedad que produce este patógeno en peces, de otras septicemias infecciosas, causadas por *Aeromonas* y *Pseudomonas* con signos patológicos similares. Las infecciones agudas, si no se detectan con rapidez, pueden ocasionar la pérdida del 30-70% del stock de peces y en regiones en las que la enfermedad es endémica, se mantiene una tasa baja pero persistente de mortalidad por yersiniosis.

Y. ruckeri se aisló inicialmente de la trucha arcoíris (*O. mykiss*) en Idaho (USA) en los años 50 (Rucker, 1966; Ross y col., 1966). Sin embargo, el origen de la enfermedad permanece incierto. A partir de la década de los 50, los aislamientos del microorganismo se incrementaron, siendo descritos por Busch (1982) como una enzootia de las principales granjas de salmónidos de USA y Canadá. Por otra parte, se cree que la ERM se ha dispersado globalmente desde USA a partir de la exportación de truchas durante la expansión de la práctica de este cultivo.

Se ha planteado la hipótesis de que la ERM pudo haber existido en Norte América, como se sugirió a partir de un aislado de *Y. ruckeri* conservado en el "National Fisheries Center," (Leewton, USA) cuya fecha de aislamiento databa antes del primer reporte realizado por Rucker en la década de los 50. También se encontró una cepa australiana con fecha de los 80's, conservada en dicho centro.

Las primeras evidencias de ERM en Europa aparecen publicadas por Lessel y col. (1983), quienes describieron un primer asilamiento de *Y. ruckeri* en Francia en 1981. Busch (1978), sostiene que algunos casos de ERM ocurrieron en Italia antes de 1978 aunque no cita ninguna referencia. Por otra parte, aunque hubo indicios de detecciones de *Y. ruckeri* en Inglaterra antes de los 80's, no fue hasta 1983 cuando se publicó el primer aislamiento a partir de truchas (Roberts, 1983). Este trabajo también incluyó la primera descripción de aislados no-motiles de *Y. ruckeri* como responsables de un brote de ERM que causó pérdidas de 200-300 truchas por día.

Posteriormente, los aislamientos de *Y. ruckeri* a partir de brotes de ERM se fueron incrementando a través de Europa y el mundo durante la década de los 80, reportándose en Dinamarca e Italia en 1983 (Dalsgaard y col., 1984; Giorgetti y col., 1985), en Noruega, España e Irlanda en 1985 (McArdle y Dooley-Martin, 1985; De la Cruz y col., 1986; Sparboe y col., 1986), y en Suiza en 1986 (Meier, 1986). Aunque Suramérica es unos de los más grandes productores de salmónidos desde los años 80, los primeros aislamientos de *Y. ruckeri* datan de 1998 con un incremento hacia el 2000 en Perú, y desde 1992 en Chile (Toledo col., 1993; Bravo y Kojahura, 2004).

Actualmente la enfermedad se extiende por las piscifactorías de salmónidos de América (USA, Canadá, Chile y Perú), Europa (Dinamarca, Francia, Italia, Alemania, Noruega, Reino Unido, España y Portugal), Australia y Sudáfrica.

1.1.1. Signos clínicos de la infección

La yersiniosis afecta a peces de todas las tallas, siendo más aguda en los alevines y presentándose en condiciones crónicas en los peces más grandes. Los cambios que se pueden observar en el comportamiento de los peces, incluyendo natación cerca de la superficie, letargo y pérdida del apetito. La evidencia experimental sugiere que el tiempo de incubación es de 5 a 10 días a 13-15 °C (Bullock y Cipriano, 1990). La severidad de la enfermedad depende principalmente de la virulencia de las cepas y el grado de estrés ambiental. Factores como aumento de la temperatura (> 20 ºC) y la salinidad (< 20‰) del agua, son determinantes en el establecimiento de infecciones de *Y. ruckeri*, (Altinok y col., 2001; Altinok, 2004).

Aunque el típico enrojecimiento de la boca (que dió el nombre a esta enfermedad) no siempre es evidente (Frerichs y col., 1985), es común observar en peces afectados hemorragias en la superficie del cuerpo, alrededor de los opérculos, en la base de las aletas y en la zona de la línea lateral, así como también el abdomen distendido (Rucker, 1966; Ewing y col., 1978) (Fig. 1.2). Tambien puede observarse exoftalmia acompañada de hemorragia orbital, que se evidencia como un aro hemorrágico alrededor del ojo y, algunas veces, puede además ocurrir edema retrobulbar (hinchazón debajo del ojo) (Horne y Barnes, 1999; Avci y Birinioğlu, 2005).

Internamente, se presentan hemorragias en la superficie del hígado, páncreas, ciegos pilóricos y vejiga natatoria (Fig. 1.3). El bazo se observa agrandado y ennegrecido. Hay acumulación de líquido abdominal e inflamación del intestino, que aparece lleno de un líquido amarillento. Otras veces, la infección

se caracteriza por la aparición de ulceraciones externas sin afectar los órganos internos (Frerichs y col., 1985; Romalde, 1992).

Figura 1.2. Peces con síntomas externos típicos de yersiniosis: hemorragia orbital y enrojecimiento de la boca (A); hemorragia en la base de las aletas, alrededor del opérculo y abdomen distendido (B). (Fotos J. L. Romalde).

Hematológicamente, algunos parámetros sanguíneos como el volumen corpuscular medio (VCM) y los niveles de proteína sanguínea total pueden mostrar un aumento en un 50% sobre los valores normales (Wobeser, 1973). Por otra parte, los valores de hematocrito, contenido de hemoglobina, recuento de leucocitos y eritrocitos disminuyen por la acción de las endotoxinas de *Y. ruckeri,* que afectan la coagulación sanguínea, produciendo trombosis en los capilares y hemorragias (Lehman y col., 1987). En peces supervivientes a la infección experimental con *Y. ruckeri* se ha determinado hipoglucemia transitoria e hipocolesterolemia debido a la disminución de la fracción albúmina en la sangre (Quentel y Aldrin, 1986).

Figura 1.3. Hemorragia en órganos internos y acumulación de líquidos causado por *Yersinia ruckeri* en peces.

La histopatología de los peces infectados muestra septicemia generalizada con respuesta inflamatoria en casi todos los tejidos, observándose mayor colonización bacteriana en los tejidos altamente vascularizados como el bazo, riñón, corazón, hígado y branquias (Rucker, 1966).

En el hígado, los principales signos son la acumulación de células mononucleares y la deformación de hepatocitos, que aparecen sin la vacuolización prominente normal. Además, se puede observar pérdida importante del tejido hematopoyético debido a la necrosis del riñón anterior y posterior (Wobeser, 1973). La presencia de la bacteria en el intestino y branquias ha sido corroborada por Fernández y col. (2003), al observar actividad β-galactosidasa en estos órganos tras realizar infecciones experimentales con cepas de *Y. ruckeri* que contenían fusiones transcripcionales con el gen *lacZ*. La utilización de la proteína fluorescente verde (GFP) ha permitido localizar la bacteria también en bazo y sangre (Welch y Wiens, 2005).

Es frecuente que se observen infecciones sin que se produzca ninguna señal aparente de la enfermedad. En este caso, los peces portadores adoptan un comportamiento letárgico, inapetencia y puede observarse oscurecimiento de la piel. En condiciones de estrés para el animal tales como el incremento de temperatura del agua, alta densidad de población, traslados, etc., es cuando tiene lugar la aparición de los brotes.

1.2. *Yersinia ruckeri*

1.2.1. *Posición taxonómica*

Yersinia ruckeri se aisló inicialmente en los años 50, caracterizándose en profundidad durante los años 60 (Ross y col., 1966; Rucker, 1966), para ser finalmente definida como especie dentro del género *Yersinia* en 1978 (Edwing y col., 1978). Este género se incluye en la familia *Enterobacteriaceae* y es conocido fundamentalmente por incluir tres especies patógenas para el hombre: *Yersinia pestis*, agente etiológico de la peste, *Yersinia enterocolitica* y *Yersinia pseudotuberculosis*, responsables ambas de procesos gastrointestinales y afecciones de ganglios mesentéricos.

Según la segunda edición del manual Bergey's (Bottone y col., 2005), el género *Yersinia* está constituido por las especies: *Y. enterocolitica* (Scheifstein y Coleman, 1943), *Y. rodhei* (Aleksic y col., 1987), *Y. ruckeri* (Ewing y col., 1978), *Y. pseudotuberculosis* (Pfeiffer, 1889), *Y. pestis* (Van Loghem, 1944), *Y. frederiksenii* (Ursing y col., 1980), *Y. bercovieri, Y. mollaretii* (Wauters y col., 1988), *Y. intermedia* (Brenner y col., 1980), *Y. aldovae* (Bercovier y col., 1984) y *Y. kristensenii* (Bercovier y col., 1981). Además, en los últimos años se han descrito otras cinco especies: *Y. aleksiciae* (Sprague y Neubauer, 2005), *Y. massiliensis* (Merhej y col., 2008), *Y. similis* (Sprague y col., 2008), *Y. nurmii* (Murros-Kontiainen y col., 2011) y *Y. entomophaga* (Hurst y col., 2011).

Y. ruckeri se ubicó dentro del género *Yersinia* en base al contenido de guanina+citocina (G+C) de su DNA (47,5-48%) (Ewing y col., 1978), difiriendo de las especies *Serratia* (52-60% G+C) y relacionándose estrechamente con otras especies de *Yersinia* (46-50% G+C). Sin embargo, su ubicación taxonómica dentro de este género sigue siendo objeto de controversia. Las similitudes

bioquímicas con el género *Serratia* y *Salmonella*, así como la reacciones serológicas cruzadas con *Hafnia alvei* y *Salmonella* sp. han hecho que varios autores consideren que este microorganismo puede llegar a constituir un nuevo género independiente (Ross y col., 1966; Llewellyn, 1980; Stevenson y Daley, 1982).

Estudios realizados utilizando técnicas de análisis de genes funcionales (MLST) señalan a *Y. ruckeri* como la especie más distante dentro del género *Yersinia* (Bottone y col., 2005; Kotetishvili y col., 2005). Por otra parte, los estudios de patogenicidad indican que los mecanismos de virulencia usados por *Y. ruckeri* son diferentes a los encontrados en otras especies del género *Yersinia* (Tooback y col., 2007).

1.2.2. *Características morfológicas y de crecimiento*

Las células de *Y. ruckeri* tienen forma de bacilos de 0,5–0,8 μm de diámetro y entre 1,0–3,0 μm de longitud (Fig. 1.4). Esta bacteria no forma esporas ni posee cápsula (Ross y col., 1966). Aproximadamente el 80% de las cepas aisladas son móviles, presentando 7 u 8 flagelos en disposición perítrica, que dejan de ser funcionales a temperaturas inferiores a 9 ºC y están ausentes a 35 ºC (Davies y Frerichs, 1989).

El microorganismo puede ser fácilmente aislado de órganos internos usando una variedad de medios de cultivos (Tabla 1.2). La bacteria crece en un rango amplio de temperatura con un óptimo de 28-29 ºC (Stevenson y col., 1993). Los cultivos celulares de más de 48 horas presentan en ocasiones largas cadenas de células. Las colonias son de color crema y tienen entre 1–1,5 mm de diámetro.

Las variaciones en las formulaciones de los medios de cultivo tienen un efecto sobre la morfología celular general de *Y. ruckeri*. Para el medio agar

tripticasa de soja (TSA) suplementado con 3% (p/v) de cloruro de sodio (NaCl), por ejemplo, se ha observado una marcada diferencia en el tamaño celular, de 0,5 µm de diámetro y de 4–6 µm de longitud. En cuanto a su hábitat, *Y. ruckeri* sobrevive en aguas débilmente saladas y en los sedimentos y forma parte de la microbiota de las aguas dulces.

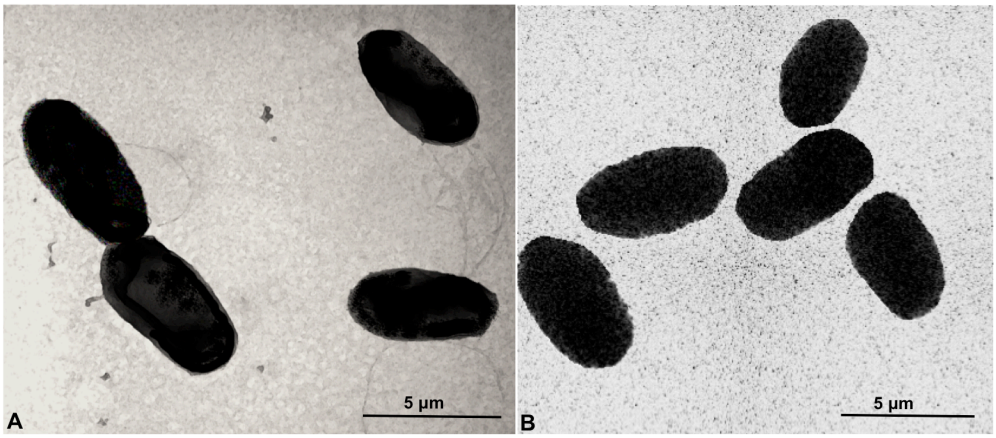

Figura 1.2. Fotografías realizadas al microscopio electrónico de transmisión de células de *Yersinia ruckeri*. A) cepa móvil (Biotipo 1) y B) cepa inmóvil (Biotipo 2).

Table 1.2. Medios usados comúnmente para el aislamiento de *Yersinia ruckeri.*

Medio	Referencia
Agar tripticasa de soja	Stevenson y Daley, 1982
Agar nutritivo	Secades y Guijarro, 1999
Agar cerebro-corazón	Arias y col., 2007
Agar sangre	Gibello y col., 2004
Agar McConkey	Gibello y col., 1999

1.2.3. *Características bioquímicas*

Y. ruckeri, al igual que otras enterobacterias, es fermentadora de glucosa, positiva para la producción de catalasa y reducción de nitratos, y negativa para la producción de oxidasa, diferenciándose de otros miembros de género *Yersinia* por la capacidad de producir lisina descarboxilasa (Ross y col., 1996). Es negativa para la pruebas de indol, salicina y esculina. La capacidad para hidrolizar la gelatina, caseína y Tween 80, así como para producir acetoína y fermentar el sorbitol varía entre las distintas cepas (Tabla 1.3), lo que permite utilizar estas características en estudios taxonómicos y epidemiológicos.

De esta manera se ha clasificado a *Y. ruckeri* en dos biotipos (1 y 2) en función de la movilidad y la capacidad para hidrolizar el Tween 80, siendo el biotipo 1 (BT1) móvil y lipasa positivo, y el biotipo 2 (BT2) negativo para ambas pruebas (Davies y Frerichs, 1989). Aunque se han descrito en la literatura diferentes características diferenciales entre las cepas inmóviles y móviles (Tabla 1.4), no hay hasta ahora un procedimiento estándar a parte de la capacidad de movilidad y de hidrólisis de Tween 80, para distinguirlas.

Tabla 1.3. Características bioquímicas de *Yersinia ruckeri.*

Prueba bioquímica	Resultado
Metabolismo fermentativo	+
Prueba de Rojo de Metilo	**V**
Reducción de nitrato	+
Reacción de Voges-Proskauer	**V**
Producción de:	
Arginina dihidrolasa	–
Catalasa	+
β–galactosidasa	+
H_2S	–
Indol	–
Lisina decarboxilasa	+
Ornitina decarboxilasa	+
Oxidasa–fenilalanina deaminasa	–
Fosfatasa	–
Degradación de :	
Esculina	–
Quitina	–
DNA	–
Elastina	**V**
Gelatina	**V**
Pectina	–
Tributirina	**V**
Tween 20	**V**
Tween 40	**V**
Tween 60	**V**
Tween 80	**V**
Urea	–
Utilización de citrato	+
Producción de ácido a partir de:	
Fructosa	+
Glucosa	+
Inositol	–
Lactosa	–
Maltosa	+
Manitol	+
Rafinosa	–
Salicina	–
Sorbitol	–
Sucrosa	–
Trehalosa	+
Xilosa	–

+, positivo; –, negativo; V, variable.

Tabla 1.4. Características bioquímicas variables entre cepas de *Yersinia ruckeri* correspondientes al biotipo 1 y biotipo 2 (Davies y Frerichs, 1989, Austin y col., 2003).

Prueba Bioquímica	Biotipo 1	Biotipo 2
Movilidad	+	–
Hidrólisis del Tween 80	V	–
Reacción Voges-Proskauer	V	+
Producción de ácido a partir de sorbitol	V	V
Prueba de Rojo de Metilo	V	V

+, positivo; –, negativo; V, variable

1.2.4. *Clasificación serológica*

Desde su primera descripción, se han establecido diferentes esquemas de tipado serológico para *Y. ruckeri,* basados en el reconocimiento de estructuras antigénicas variables, como antígenos somáticos "O" (lipopolisacáridos; LPS), proteínas de membrana externa (OMPs) y antígenos flagelares "H".

Los primeros aislados de *Y. ruckeri* en USA mostraban características antigénicas uniformes cuando eran estudiados usando tanto antisueros frente a antígenos O, como a antígenos H (Ross y col., 1966; Bush, 1973). En 1977, un estudio serológico con cepas de *Y. ruckeri* aisladas de salmón del pacífico, usando antisueros con célula entera, evidenciaron la presencia de antígenos mayoritarios diferentes a los de la cepa típica o cepa Hagerman (O'Leary, 1977). Luego de la confirmación específica de muchas de estas cepas mediante hibridación de DNA, se reconoció una segunda serovariedad en *Y. ruckeri* (De Grandis y col., 1988).

En 1980, una bacteria ("salmonid blood spot bacterium") aislada del salmón del Atlántico en Australia, se identificó como *Y. ruckeri* mediante hibridación de DNA. Este aislado mostró una reacción de aglutinación cruzada con el aislado original (serovariedad I), siendo designada como serovariedad I' (Stevenson y Ardrie, 1984b). Posteriormente, otra cepa de Australia encontrada en la colección de cultivos de la "United States Fish and Wildlife Service National Fish Health Laboratory" (Kearneysville, USA) se designó como serovariedad III (Bullock y col., 1978), aunque posteriormente se demostró que las serovariedaes I' y III eran idénticas (Daly y col., 1986). En 1986 ya se habían identificado 5 serovariedades en *Y. ruckeri*, a partir de aislados de peces enfermos y controles rutinarios de monitoreo, mediante el uso de pruebas de aglutinación utilizando células completas (Stevenson y Airdrie, 1984b; Daly y col., 1986).

De Grandis y col. (1988) demostraron que las cepas de las serovariedades I, II y III (incluidas en el serotipo O1) así como las V y VI podían considerarse claramente como *Y. ruckeri*, pero que las cepas de la serovariedad IV y otras que no habían sido tipadas en ninguna de estas serovariedades probablemente se correspondían con *Hafnia alvei.*

Flett (1989) utilizando los perfiles de LPS y sus reacciones inmunológicas mediante técnicas de "immunoblot", identificó 6 serotipos "O" en *Y. ruckeri*. De esta manera, las serovariedades I y III constituyeron el serotipo O:1, ya que mostraron el mismo perfil de LPS y reacción inmunológica. La serovariedad II presentó características variables subdividiéndose en tres serotipos separados (O:2, O:3 y O:4). Las cepas asignadas a las serovariedades V y VI también presentaron patrones de LPS únicos y se identificaron como serotipos O:5 y O:6 respectivamente.

Davies (1990) establece un nuevo esquema de tipificado definiendo cinco O-serotipos (O1, O2, O5, O6 y O7) utilizando antígenos termoestables de 127 cepas de *Y. ruckeri* aisladas de Europa y Norteamérica. Este autor sugiere que el aislado australiano previamente descrito como serovariedad III se había tipado incorrectamente y que en realidad correspondía al serotipo O1.

En 1993, Romalde y col. (1993) basándose en el estudio de antígenos-O termoestables, LPS y proteínas de membrana externa (OMP), proponen un nuevo esquema de serotipado distinguiendo cuatro serotipos. De este modo, el seroripo O1 se subdivide en dos subgrupos: O1a (anterior serovariedad I) y O1b (anterior serovariedad III). El serotipo O2 (equivalente a la serovariedad II) se divide en tres subgrupos O2a, O2b y O2c. Los serotipos restantes se designan como O3 (serovariedad V) y O4 (serovariedad VI). La Tabla 1.5 muestra una comparación entre estos sistemas de serotipado. Entre estos diferentes serotipos, la clásica serovariedad I, equivalente al actual serotipo O1a (Romalde y col., 1993), comprende las cepas más virulentas y responsables de la mayoría de los brotes de yersiniosis descritos en el cultivo de salmónidos.

Los perfiles de OMP también se han utilizado para clasificar los aislados de *Y. ruckeri*, identificándose 5 tipos diferentes (Davies, 1990). El tipo de OMP es determinado por la presencia de una proteína termo-modificable (HMP) de 36 ó 38 kDa y de una proteína asociada a un péptidoglicano (PAP) con peso molecular entre 36,5 y 40,5 kDa. A partir de estos estudios, se propuso un esquema para agrupar las cepas de *Y. ruckeri* en base a la combinación del biotipo, serotipo y perfil OMP, útil para discriminar entre diferentes cepas de *Y. ruckeri*, demostrar la relaciones entre aislados e identificar diferentes grupos clonales (Davies, 1991a). Así, la serovariedad I se dividió en seis grupos clonales de los cuales sólo dos se asociaron a la ocurrencia de la mayoría de las mortalidades. El grupo clonal 5 que

comprendió la mayoría de los aislados móviles y se encontró distribuido ampliamente, y el grupo clonal 2 que incluyó sólo cepas inmóviles.

Tabla 1.5. Equivalencia entre diferentes sistemas de tipado serológicos propuestos para *Yersinia ruckeri.*

Serovariedades Clasificación clásica[a]	**Serovariedades** De Grandis y col. (1988)	**Serotipos** Flett (1989)	**Serotipos** Davies (1990)	**O-serotipos** Romalde y col. (1993)
I	I	O1	O1	O1a
II	II	O2 O3 O4	O2	O2a O2b O2c
III	III	O1	O1	O1b
IV	No *Y. ruckeri*	–	–	–
V	V	O5	O5	O3
VI	VI	O6	O6	O4
NO TIPADAS	No *Y. ruckeri*	–	O7	–

[a] Establecido entre 1966 y 1986 por: Ross y col., 1966; Bullock y col., 1978; O´Leary y col., 1979; Stevenson y Airdrie, 1984b; Daly y col., 1986.

1.2.5. *Caracterización genética*

Los avances tecnológicos en el diagnóstico molecular han conducido a que el tipado molecular se haya convertido actualmente en un procedimiento rápido y sencillo, mientras que anteriormente estas técnicas eran complejas y largas. En general, el tipado molecular se ha utilizado para caracterizar patógenos bacterianos de seres humanos y animales, incluyendo peces y otros organismos acuáticos.

La variabilidad fenotípica de *Y. ruckeri* dificulta relacionar los brotes de ERM con una fuente potencial porque éstas características se expresan de manera inconsistentes. No obstante, el uso de técnicas de tipado molecular y de diagnóstico tales como análisis electroforético de múltiples locus enzimáticos (MLEE), electroforesis en campo pulsado (PFGE), el análisis de secuencias consenso intergénicas repetitivas (ERIC-PCR) y de elementos palindrómicos extragénicos repetitivos (REP-PCR), han permitido entender algunos aspectos sobre la epidemiología de este microorganismo.

El MLEE fue una de las primeras técnicas utilizadas para estudiar genéticamente a *Y. ruckeri* sobre una escala de tiempo usando una amplia variedad de aislados. Schill y col. (1984) utilizando MLEE encontraron sólo cuatro tipos electroforéticos entre 47 cepas, y definieron a *Y. ruckeri* como una especie con una alta homogeneidad a nivel genético, sugiriendo que la población era clonal. Sin embargo, Davies (1990) mediante el uso de la técnica de PFGE, logró distinguir entre los serotipos de *Y. ruckeri,* encontrando además, diferentes pulsotipos en cepas fenotípicamente similares, lo que supuso que podrían haber surgido por separado. Romalde y col. (1991) también encontraron heterogeneidad entre los diferentes serotipos utilizando técnicas variantes de PFGE, tales como electroforesis en gel de campo invertido (FIGE) y en campo eléctrico homogéneo en contorno cerrado (CHEF). Recientemente, Wheeler y col. (2009), realizaron un amplio estudio en 160 aislados de *Y. ruckeri* utilizando también la PFGE, obteniendo 44 pulsotipos diferentes, evidenciando una mayor variabilidad genética de la especie.

El análisis de los perfiles plasmídicos y de los patrones de ribotipado también ha sido útil en el estudio de *Y. ruckeri*. Romalde (1992) observó variabilidad entre cepas sin poder establecer correlación entre la presencia de un

plásmido determinado y la presencia de un serotipo particular. García y col. (1998) encontraron en *Y. ruckeri* hasta 8 perfiles plasmídicos y 11 ribotipos diferentes al analizar un total de 183 cepas aisladas de diferentes lugares geográficos. La predominancia de un perfil en ambas técnicas hizo sugerir también una estructura clonal en la especie. La mayoría de las cepas del serotipo O1 presentaban un plásmido de gran tamaño (75 MDa) cuya implicación en la virulencia todavía no ha sido determinada. Lo que sí está claro es que no presenta homología con los plásmidos de virulencia de otras especies patógenas del género *Yersinia* (Guilvout y col., 1988). Sin embargo, Sousa y col. (2001), utilizaron diferentes técnicas de tipado molecular y obtuvieron mayor discriminación entre diferentes aislados portugueses en base al análisis de los patrones de ribotipado, logrando diferenciar las cepas según la piscifactoría de origen y/o la época de aislamiento.

Otras técnicas moleculares como ERIC-PCR han sido usadas para estudiar la diversidad genética entre aislados ambientales de *Y. ruckeri*, distinguiéndose apenas diferencias en la presencia de tres fragmentos (721, 912 y 1.025 pb) en tres de 22 aislados estudiados (Coquet y col., 2002). Desafortunadamente, en *Y. ruckeri* la técnica de ERIC-PCR se ha utilizado solamente como una herramienta con fines epidemiológicos en algunas ocasiones y a un nivel nacional (Coquet y col., 2002; Arias y col., 2007). Recientemente, Johns (2010) utilizó la técnica de electroforesis en gel de gradiente desnaturalizante (DGGE) para generar perfiles utilizando los genes 16S rRNA, *gln*A y *gyr*B de *Y. ruckeri,* encontrando un patrón idéntico en todas las cepas. Por otra parte, los patrones generados usando DGGE con fragmentos de ERIC-PCR y REP-PCR, evidenciaron una subdivisión de los aislados estudiados, pero no se logró establecer ninguna relación con los serotipos o fenotipos.

La mayoría de los métodos mencionados anteriormente están basados en la generación de patrones de bandas en geles de agarosa y su comparación en formato de imagen. Así pues, con el empleo de estos métodos, compartir información es un proceso complejo que requiere la formación de redes amplias que permitan la estandarización del método y la implantación de idéntica metodología para el análisis e intercambio de imágenes (Canhos y col., 2004; Swaminathan y col., 2001). La técnica de tipado de secuencias multilócicas (MLST) utiliza un enfoque basado en la secuencia de nucleótidos de genes esenciales ("housekeeping genes", HK), que puede aliviar todos los inconvenientes mencionados de las técnicas anteriormente descritas. Este enfoque fue desarrollado por Maiden y col. (1998) para caracterizar las infecciones meningocócicas, y ha sido adoptado para estudiar muchos otros patógenos bacterianos.

El número de variantes detectados es mucho mayor utilizando la estrategia directa de secuenciación en vez de la indirecta de movilidad electroforética. Se ha calculado que son necesarios hasta 26 cambios en la secuencia del gen para producir un cambio en la movilidad electroforética de la enzima codificada (Boyd y col., 1994). Mientras, el análisis de secuencias permite detectar variantes que supongan tan sólo un cambio en una base del gen analizado. Aunque el número de polimorfismos en nucleótidos de genes HK es bajo, la concatenación de las secuencias de varios de estos genes proporciona una alta capacidad de discriminación y de información sobre la evolución a largo plazo. Los beneficios de usar el MLST como un método de tipado es que los resultados son altamente reproducibles entre laboratorios y los genes utilizados están presentes en todos los aislados.

Estudios anteriores se han centrado en la definición de esquemas de MLST para el género *Yersinia*. Sin embargo, Kotetishvili y col. (2005) incluyeron sólo cuatro cepas de *Y. ruckeri* aisladas en Bélgica en un periodo de tres años, haciendo difícil una interpretación significativa sobre esta especie.

Los avances tecnológicos en el diagnóstico molecular han conducido a una mayor comprensión de las relaciones genéticas entre las bacterias y en el caso de *Y. ruckeri*, el uso de algunas técnicas moleculares han permitido evidenciar que existe diversidad intraespecífica. Este hecho, justificaría futuros estudios sobre la filogénia, diversificación y estructura poblacional de este patógeno.

1.3. Transmisión y reservorios

La expansión de la enfermedad ocurre principalmente por transmisión horizontal (de pez a pez) a través del agua (Rucker y col., 1966). Sin embargo, el aislamiento de *Y. ruckeri* a partir de mamíferos y otros animales salvajes como aves, invertebrados y hasta humanos, ponen de manifiesto la posible existencia de vectores (Hunter y col., 1980).

Y. ruckeri es capaz de permanecer viable en aguas débilmente saladas (0-20 ppt) durante, al menos 4 meses mientras, que en agua salobre (35 ppt) la viabilidad es mucho menor (Thorsen y col., 1992). Esta capacidad de supervivencia de *Y. ruckeri* fuera del hospedador durante largos periodos de tiempo, permaneciendo infectiva en los ambientes acuáticos, facilita la transmisión ya que puede llegar a causar eventualmente, nuevos brotes de la enfermedad. Romalde y col. (1994) comprobaron que la bacteria puede sobrevivir en un estado “durmiente” durante periodos de escasez de nutrientes, conservando las células la capacidad de infección.

La aparición de yersiniosis en nuevas áreas geográficas se ha atribuido a la importación de peces portadores. El reconocimiento de más hospedadores y áreas geográficas para *Y. ruckeri* se debe en parte, al incremento de la vigilancia y la mejora de los métodos para su detección, a la vez que puede estar relacionado con el incremento de nuevas instalaciones acuícolas en todo el mundo (Tooback y col., 2007).

Aunque la infección con este agente patógeno afecta principalmente a peces salmónidos, también se ha descrito en otras especies de diversas familias. En la Tabla 1.6, se resumen diferentes fuentes de aislamiento descritas para *Y. ruckeri*, incluyendo peces salmónidos y no salmónidos así como posibles vectores y otras fuentes ocasionales. Particularmente, el microorganismo ha sido aislado de peces salvajes como el lucio (*Esox lucius*), la anguila (*Anguilla anguilla*), el gobio (*Gobio gobio*), y el esturión (*Acipenser sturio*). Además, también se ha encontrado presente en otros peces marinos de interés comercial como el rodaballo *(Scophthalmus maximus),* el carbonero (*Pollachius* sp.), el lenguado (*Solea solea*) y la dorada (*Sparus aurata*) (Michel y col., 1986).

El papel del estado portador de los peces es también importante durante las infecciones de *Y. ruckeri*. Por ejemplo, en Canadá el primer aislamiento de *Y. ruckeri* se describió a partir de infecciones en truchas por peces asintomáticos (Wobeser, 1973). También se han aislado cepas de peces ornamentales como la carpita cabezona (*Pimephales promelas)* importadas a Francia y Bélgica desde USA. La principal fuente de infección es la dispersión de gran número de bacterias a través de las heces de peces portadores o enfermos (Busch y Lingg, 1975). No obstante, el número de bacterias diseminado no suele ser suficiente para causar la aparición de brotes. El problema a gran escala se presenta cuando los peces infectados son expuestos a condiciones de estrés tales como, altas

densidades y calidad pobre del agua (Hunter y col., 1980; Hornes y Barnes, 1999; Austin y Austin, 2007).

La capacidad de *Y. ruckeri* de formar biopelículas en superficies y sedimentos representa también una fuente de infección recurrente en las piscifactorías (Coquet y col., 2002; Vendrell y col., 2009). Las bacterias utilizan pili o flagelos para moverse a lo largo de la superficie para encontrar otras bacterias y formar o ampliar microcolonias. Además, las bacterias formadoras de biopelículas han demostrado ser muy resistentes al ácido oxolínico, un antibiótico de uso frecuente en el tratamiento de yersiniosis (Coquet y col. 2002).

El aislamiento del patógeno a partir de huevos de salmón desinfectados no fertilizados y la aparición de bajas tasas de mortalidad después de la fertilización, así como tras 12 semanas de alimentación, sugieren la posible existencia de transmisión vertical para *Y. ruckeri* (Sauter y col., 1985). Sin embargo no se han realizado estudios sobre esta vía de transmisión.

Tabla 1.6. Especies a partir de las cuales se han descrito aislamientos de *Yersinia ruckeri.*

FUENTE DE AISLAMIENTO	PAÍS	REFERENCIA[a]
Peces salmónidos:		
Oncorhynchus mykiss (Trucha arcoíris)	USA	Ross y col. (1996)
O. kisutch (Salmón plateado)	USA	McDaniel (1971)
O. tshawystscha (Salmón "Chinook")	USA	McDaniel (1971)
O. nerka (Salmón "Sockeye")	USA	Dulin y col. (1976)
Salmo clarkii (Trucha "Cutthroat")	USA	McDaniel (1971)
S. gairdneri (Trucha "Steelhead")	USA	McDaniel (1971)
S. salar (Salmón del Atlántico)	Canadá	McDaniel (1979)
S. trutta (Trucha común)	Canadá	McDaniel (1979)
Salvelinus fontinalis (Trucha salvelino)	Canadá	McDaniel (1979)
S. malma ("Sea trout")	Canadá	McDaniel (1979)
Thymallus thymalus (Tímalo)	Italia	Michel y col. (1986)
Peces no salmónidos enfermos:		
Notemigonus atherinoides ("Smerald shiners")	USA	Mitchum (1981)
Coregonus artedii (Cisco)	Canadá	Stevenson y Daly (1982)
Pimephales promelas ("Minnow")	Francia	Michel y col. (1986)
Coregonus peled ("Withefish")	Finlandia	Rintamaki y col. (1986)
C. muksum ("Withefish")	Finlandia	Rintamaki y col. (1986)
Acipenser sturio (Esturión)	Francia	Vuillaume y col. (1987)
Scophthalmus maximus (Rodaballo)	Francia	Michel y col. (1986)
Sparus aurata (Dorada)	Francia	Michel y col. (1986)
*Esox lucius (*Lucio*)*	Alemania	McDaniel (1971)
Anguilla anguilla (Anguila)	Francia	Fuhrmann y col. (1984)
Gobio gobio (Gobio)	Francia	Vuillaume y col. (1987)
Pollachius sp. ("Coalfish")	Francia	Michel y col. (1986)
Soleidae sp. (Lenguados)	Canadá	Vuillaume y col. (1987)
Rutilus rutilus (Rutilo)	Irlanda	McDaniel (1979)
Carassius auratus ("Goldfish")		McArdle y Dooley-Martyn (1985)
Vectores:		
Larus spp. (Gaviotas)	Noruega	Willumsem (1989)
Pollachius virens (Abadejo)		Willumsem (1989)
Otras fuentes accidentales:		
Ondatra zibethica (Rata de agua)		Stevenson y Daly (1982)
Heces de aves rapaces (Buitres)		Bangert y col. (1988)
Penaeus keraturus (Langostino)		Sullivan (1981)
Invertebrados acuáticos		Austin y Austin (2007)
Hombre		Farmer y col. (1985)

[a] Descripciones del primer aislamiento.

1.4. Patogenia

La investigación sobre los mecanismos de patogenicidad en *Y. ruckeri* es muy limitada, probablemente debido al control eficaz de la ERM durante muchos años mediante la vacunación. Se conoce mucho más acerca de las interacciones huésped-bacteria de las tres especies de *Yersinia* que causan enfermedades en los seres humanos: *Y. pestis*, *Y. pseudotuberculosis* y *Y. enterocolitica*. Debido a esto, se ha tratado de conocer los mecanismos de patogenicidad en *Y. ruckeri*, investigando la presencia de factores de virulencia comunes con las especies patógenas de humanos. A pesar del hecho de que estos agentes causan diferentes signos clínicos y utilizan diferentes modos de transmisión, se han identificados varios mecanismos de virulencia comunes.

1.4.1. *Ruta de entrada*

El conocimiento sobre el portal de entrada es importante en la patogénesis bacteriana, ya que proporciona información sobre la etapa inicial de desarrollo de la enfermedad y puede conducir al desarrollo de estrategias terapéuticas eficaces. Las branquias, el intestino y la piel se han identificado como importantes vías de infección de patógenos peces. Todos estos tejidos se cubren con una capa mucosa, representando la primera barrera física que tiene que ser atravesada por el patógeno para iniciar la infección. Se ha encontrado que algunos agentes patógenos se adhieren eficazmente a los componentes del mucus, tales como mucina y glicoconjugados, penetran a través de la capa mucosa y alcanzan el epitelio subyacente (Chen y col., 2008). Sin embargo, el alcance de la actividad antibacteriana de esta capa mucosa parece variar entre especies de peces y, en algunos casos, puede ser específico para el microorganismo (Svendsen y Bogwald, 1997).

Por otra parte, las branquias están en contacto constante con el medio acuático y, por tanto, representan un acceso directo de ataque para los patógenos. Las bacterias pueden penetrar en el epitelio branquial que, por estar altamente vascularizado, puede proporcionar un buen sitio de entrada del patógeno y su fácil difusión a través de todo el cuerpo de los peces (Ling y col., 2001.). Estructuralmente, la piel es una barrera natural más gruesa y compacta que las branquias o el intestino. Algunos patógenos son capaces de entrar en la piel intacta, aunque claramente la existencia de lesiones facilita la invasión (Spanggaard y col., 2000). Se ha demostrado que después de la adhesión, las bacterias patógenas logran penetrar progresivamente en los tejidos más profundos y en los músculos (Smith y col., 2003).

En el caso *Y. ruckeri* se ha observado que el intestino puede ser una importante puerta de entrada del microorganismo al hospedador (Busch y Lingg, 1975; Valtonen y col., 1992). La colonización de este órgano puede ocurrir a través del consumo de alimentos o agua contaminados. La penetración epitelial o endocitosis del patógeno puede aumentar la invasión a la sangre y al sistema linfático, facilitando la propagación del patógeno a través del cuerpo de los peces. La intubación intestinal experimental de patógenos también se ha mostrado eficaz para producir infección y mortalidad (Olsson y col. 1996; Smith y col., 2003). En recientes estudios se ha descrito que la progresión de *Y. ruckeri* durante el proceso infeccioso en trucha arcoíris depende de la vía de administración de las bacterias. Así, cuando la infección se realiza por inmersión, el primer órgano afectado es el tracto digestivo desde el cual la bacteria se disemina al resto del pez (Méndez, 2012).

Se ha demostrado que las rutas de entrada más efectivas de *Aeromonas salmonicida* en la trucha arcoíris son las branquias y el ano (Efendi y Austin,

1995). En otros estudio se ha encontrado que la bacteria *V. anguillarum* puede inducir vibriosis en la anguila y en el pez ayu (*Plecoglossus altivelis*) cuando es inoculada por intubación anal (Kanno y col., 1989; Marco-Noales y col., 2001). Sin embargo, la entrada de las bacterias hacia el intestino por la abertura anal, en condiciones naturales, aún no ha sido demostrada. Esta condición requeriría además la infección contra el flujo natural de los contenidos intestinales.

1.4.2. *Virulencia*

La virulencia de un microorganismo se expresa comúnmente en términos de dosis letal media (DL_{50}) (Davis y col., 1981). El grado de virulencia de los aislados de *Y. ruckeri* es variable y puede estar influenciado entre otros por el procedimiento experimental empleado, la ruta de inoculación, así como por la susceptibilidad de la especie de pez y poblaciones utilizadas. No obstante, las mortalidades suelen ocurrir entre 5 y 10 días después de la inoculación intraperitoneal con una dosis de 10^6 células/pez (Avci y Birincioğlu, 2005). Raida y Buchman (2008) determinaron en trucha arcoíris una DL_{50} de 5 x 10^5 unidades formadoras de colonia (ufc)/pez, en inoculaciones por inyección intraperitoneal, y de 1 x 10^7 ufc/ml en los peces inoculados por inmersión. Los experimentos iniciales llevados a cabo utilizando cepas del BT2 de *Y. ruckeri* sugieren una mayor virulencia de estos aislados con LD_{50} de 5 x 10^2 ufc/pez en inoculaciones intraperitoneales (Fouz y col., 2006). Por otro lado se ha descrito que variaciones en la talla de los peces entre 5 y 60 g, no tiene efecto alguno sobre la virulencia (Furones y col., 1990).

La recurrencia de brotes de ERM en peces vacunados, ha resaltado la necesidad de estudiar los mecanismos de virulencia de esta bacteria en busca de

mejores estrategias de prevención y tratamiento. A continuación, se describen varios factores que han sido relacionados con la patogenicidad de esta bacteria.

1.4.3. *Factores relacionados con la patogenicidad*

1.4.3.1. Adherencia e invasividad

La adherencia bacteriana a los tejidos del hospedador es el paso inicial para el establecimiento del patógeno y es fundamental para la colonización y posterior progresión de la infección mediante liberación de toxinas e invasión celular (Mantle y Husar, 1993). Se ha demostrado que las células de *Y. ruckeri* son capaces de adherirse a diferentes glicoproteínas del mucus intestinal de la carpa (Schroers y col., 2005). La capacidad de adherencia de *Y. ruckeri* a diferentes líneas celulares de peces también se ha estudiado, aunque solo pudo observarse una ligera capacidad de adherencia e invasión por parte de algunas cepas a la línea celular CHSE-214 de embrión de salmón (Romalde y Toranzo, 1993).

Y. enterocolitica y *Y. pseudotuberculosis* producen al menos tres proteínas de función adherente: la invasina y la proteína Ail que son codificadas cromosómicamente, y la proteína YadA codificada en un plásmido de virulencia de 70 kb. La invasina y la proteína YadA son dos adhesinas de membrana externa que se unen a las integrina β1 de las células del hospedador. La unión de las bacterias a través de estas adhesinas parece ser importante para promover la internalización en macrófagos, así como en neutrófilos y células dendríticas (Hudson y col., 2005). La proteína Ail (de membrana externa) también desempeña un papel en la invasión de células, aunque su receptor de unión es desconocido. Kawula y col. (1996) no encontraron ninguna evidencia de genes homólogos *inv* o *ail* en *Y. ruckeri* aunque debe tenerse en cuenta que solo usaron una cepa. Sin embargo, Fernández y col. (2007) utilizando PCR y análisis de secuenciación,

sugirieron que *Y. ruckeri* posee un gen *inv* homólogo que podría estar involucrado en adherencia bacteriana y la invasión en las células del huésped.

No se ha realizado ninguna investigación exhaustiva sobre la capacidad de *Y. ruckeri* para sobrevivir dentro de los macrófagos. Sin embargo, Welch y Wiens (2005) observaron un pequeño número de bacterias intracelulares en el riñón, bazo y fagocitos de sangre periférica de trucha arcoíris, después de la inmersión y la infección intraperitoneal con una cepa de *Y. ruckeri* mutada para expresar la proteína GFP.

1.4.3.2. Mecanismos de regulación y expresión genética

Durante el desarrollo del proceso infeccioso, la bacteria emplea diferentes mecanismos de regulación para controlar la expresión de determinados genes (especialmente relacionados con la patogenicidad) y le permiten adaptarse al nuevo ambiente que encuentra en el interior del hospedador.

El hierro es una molécula importante para la supervivencia bacteriana. Este es un requisito absoluto para el crecimiento de las bacterias, las cuales lo obtienen de las proteínas del huésped, mediante los sideróforos (Neilands, 1995). Los sideróforos pueden ser divididos en tres clases principales, catecoles, hidroxamatos y compuestos heterocíclicos. El sideróforo tipo catecol ruckerbactina es el sistema de captación de hierro de *Y. ruckeri,* y ha sido involucrado con la virulencia de la misma (Fernández y col., 2004). El hierro está generalmente unido fuertemente a las moléculas, tales como la hemoglobina y la transferrina. Después de ser secretados, los sideróforos secuestran y solubilizan el hierro y lo transportan al interior de la célula través de diversos receptores para ser utilizado en diferentes vías metabólicas (Faraldo-Gómez y Sansom, 2003).

Anteriormente, la presencia de varias proteínas en la membrana externa de la bacteria había sido asociada con el crecimiento de ésta en condiciones

restrictivas de hierro (Romalde y col., 1991). Otros autores han postulado que estas proteínas actuarían como receptores para los sideróforos (Davies, 1991b; Fernández y col., 2004). La estructura química y la ruta biosintética del sideróforo ruckerbactina aún no se han determinado. No obstante, el análisis de secuencias reveló que el receptor de la ruckerbactina tenía la mayor homología con el receptor de la ferricrisobactina del patógeno de plantas *Erwinia chrysanthemi*, y presentaba más similitud con receptores de sideróforos de tipo hidroxamato que con receptores de catecoles (Fernández y col., 2007). La expresión los genes implicados en la síntesis de sideróforos de *Y. ruckeri* aumenta durante la infección de los peces mostrando valores superiores a temperatura de 18 °C.

El sistema de transducción de señales de dos componentes BarA/UvrY está constituido por una proteína quinasa de histidina BarA y una proteína reguladora de respuesta UvrY. Este sistema en *Escherichia coli* y en sus homólogos en otras bacterias Gram negativas, tales como BarA/SirA en *Salmonella*, ExpS/ExpA en *Erwinia*, VarS/VarA en *Vibrio* y GacS/GacA en diferentes especies de *Pseudomonas*, controlan positivamente la expresión de RNAs no codificantes, CsrB (RsmY) y CsrC (RsmZ). Estos pequeños RNAs actúan secuestrando proteínas represoras de traducción y activando la expresión de genes que normalmente estarían bloqueados por estos represores. Los genes activados normalmente están implicados en el metabolismo secundario, la formación de biopelículas y la motilidad. El sistema BarA-UvrY contribuye también a la patogénesis de *Y. ruckeri* en trucha arcoíris, regulando la invasión de células epiteliales y la sensibilidad al estrés oxidativo inducido por las células del sistema inmune (Dahiya y Stevenson, 2010).

La implicación en la virulencia de sistemas de captación de zinc también ha sido descrita en varios microorganismos como *E. coli* o *Salmonella entérica*

(Ammendola y col., 2007; Sabri y col., 2009; Petrarca y col., 2010; Gabbianelli y col., 2011). Las limitaciones de zinc en el hospedador pueden causar efectos pleiotrópicos en un patógeno, incluyendo expresión de genes de virulencia y supervivencia en el hospedador (Amendola y col., 2007; Crane y col., 2007).

En *Y. ruckeri* también se han identificado los componentes ZnuA (proteína periplasmática de unión al zinc), ZnuB (permeasa de membrana interna) y ZnuC (ATPasa), homólogos al transportador ZnuABC de *E. coli* (Dahiya y Stevenson, 2010). Estos autores observaron que el operón *znuABC* codifica un sistema de transporte de alta afinidad por el zinc y está implicado en el establecimiento y mantenimiento del proceso infeccioso en trucha arcoíris.

En otro estudio comparativo realizado entre cepas virulentas y no virulentas del serotipo O1, se detectó una relación entre la virulencia y la presencia de un factor sensible a la temperatura (HSF) (Furones y col. 1990). Estos autores sugirieron que el factor podría estar asociado a la envoltura celular, de manera que, los antígenos y estructuras de superficie quedarían enmascarados, lo que facilitaría la supervivencia y multiplicación de la bacteria durante el proceso infeccioso.

1.4.3.3. Toxinas extracelulares

La mayoría de los factores de virulencia extracelulares bacterianos son enzimas que facilitan la colonización y crecimiento del patógeno (Madigan y Martinko, 2006). Las proteasas extracelulares tienen como función principal suministrar nutrientes en forma de péptidos o aminoácidos al microorganismo, sin embargo, pueden también contribuir a la infección y al desarrollo de la patogénesis, por lo que se han considerado como factores de virulencia bacterianos.

Entre los productos extracelulares (ECP) de *Y. ruckeri* se han descrito

proteasas, lipasas y hemolisinas. Se ha demostrado que los ECPs de esta bacteria son altamente tóxicos, y ocasionan áreas necróticas y hemorrágicas características de la enfermedad cuando se inyectan intraperitonealmente en peces (Romalde y Toranzo, 1993). La metaloproteasa Yrp1 de 47 kDa de *Y. ruckeri* es capaz de digerir diferentes proteínas de la matriz y del músculo (como la laminina), lo que estaría directamente asociado con la capacidad de invasión de tejidos. Por otra parte, puede conducir a alteraciones de membrana y poros en los vasos capilares causando las hemorragias típicas de la ERM, especialmente alrededor de la boca y el intestino. Esta proteasa es secretada por un sistema de transporte ABC de tipo I constituido por tres proteínas (YrpD, YrpE e YrpF) y un inhibidor de proteasa Inh (Fernández y col., 2002). La presencia de actividad metaloproteasa Yrp1 no es una características general en todas las cepas de *Y. ruckeri*, y no está relacionada con el serotipo. Así, se han definido dos grupos de acuerdo con la presencia (Azo+) o ausencia (Azo–) de esta actividad proteolítica utilizando azocaseína como sustrato (Secades y Guijarro, 1999). El análisis de ambos grupos mostró que todas las cepas contienen el operón *yrp*1, pero una investigación posterior reveló que éste operón está regulado a nivel transcripcional. El fenotipo Azo– es debido a un operón *yrp*1 transcripcionalmente inactivo, o un nivel transcripcional muy bajo, que no es suficiente para la detección de la actividad proteolítica. (Fernández y col., 2003).

La expresión de la proteasa Yrp1 está influenciada por las condiciones ambientales, principalmente por la osmolaridad y la temperatura. La producción de la proteasa disminuye con el aumento de la presión osmótica del medio. Además se ha observado que la expresión del operón *yrp*1 es más alta a 18 °C y está reprimida a 28 °C, que es la temperatura óptima de crecimiento de *Y. ruckeri* (Fernández y col., 2003). Así, la proteasa Yrp1 es altamente expresada a

temperaturas que se encuentran en el huésped y puede ser una adaptación a las condiciones óptimas de temperatura para una colonización infección.

Las hemolisinas son otros de los productos extracelulares importantes, ya que la mayoría de ellas también tienen actividad citotóxica. La hemolisina/citolisina YhlA de *Y. ruckeri* es capaz de lisar los eritrocitos y las líneas celulares peces, por lo que puede estar relacionada con las propiedades invasivas como se ha demostrado para algunas toxinas de *Serratia*. En muchos microorganismos se ha demostrado que la producción de hemolisinas está además relacionada con la captación de hierro (Poole y col., 1988). Se han descrito dos genes involucrados en la producción de YhlA. El gen *yhl*B que está implicado en la secreción y activación de la hemolisina que es codificada por el gen *yhl*A (Fernández y col., 2007). Ambos genes mostraron una alta homología con genes codificantes de toxinas en *Serratia* que son secretadas por un sistema de secreción pareado también llamado sistema de secreción tipo V (T5SS). El análisis genómico reveló la presencia de hemolisinas similares en yersinias patógenas de humanos, sin embargo, su función aún no se ha determinado. A igual que el operon *yrp*1, la expresión de *yhl*A es notablemente superior a 18 °C que a 28 °C, y también bajo condiciones limitantes de hierro, lo que sugiere que estos dos factores pueden ser señales ambientales que el patógeno utiliza para producir toxinas extracelulares.

1.4.3.4. Plásmidos

Se ha encontrado que muchas cepas de *Y. ruckeri* pertenecientes al serotipo O1, que presentan un plásmido de aproximadamente 75 MDa, son resistentes a la acción bactericida de macrófagos de peces (Stave y col., 1987, Guilvout y col., 1988; Romalde y col., 1993). García y col. (1998) estudiaron el perfil de plásmidos de 183 cepas de *Y. ruckeri* de diferentes orígenes, detectando ocho

perfiles diferentes. La presencia de este gran plásmido (75 MDa) era independiente del origen geográfico de los aislados y sólo se observó en cepas de serotipo O1. Estos autores encontraron además otros plásmidos pequeños entre los diferentes serotipos, incluido el serotipo O1. Sin embargo, los plásmidos pequeños han recibido menos atención que los grandes, probablemente debido a la los intentos de correlacionar estos últimos con el plásmido de virulencia de especies de *Yersinia* patógenas para humanos. Estas especies portan un plásmido de virulencia de 70-MDa que codifica un sistema de secreción tipo III (T3SS), requerido para contrarrestar la respuesta inmune del hospedador y para asegurar la supervivencia. Específicamente, este sistema T3SS permite la comunicación directa entre las bacterias y las células huésped mediante la inyección de proteínas efectoras (Yops) en el citosol y de esta manera la bacteria modula las funciones de la célula para su propia ventaja.

Varios estudios se han centrado en la función bioquímica de estas Yops, las rutas de las señales que modulan y su papel en la patogénesis de *Yersinia* (Cornelis, 1998; Fällman y Gustavsson, 2005). En general, las principales funciones asignadas a las Yops para contrarrestar la inmunidad del huésped incluyen la destrucción de fagocitos, la supresión de la producción de citoquinas proinflamatorias y la inducción de la apoptosis de macrófagos. Guilvout y col. (1988) compararon el perfil de plásmido 18 cepas de *Y. ruckeri,* de origen estadounidense y francés, con los patrones de *Y. pestis, Y. pseudotuberculosis* y *Y. enterocolitica*. El plásmido grande de *Y. ruckeri* fue significativamente diferente al plásmido de virulencia asociado con las especies patógenas de *Yersinia* en humanos.

1.4.3.5. Componentes de envoltura celular

La envoltura de bacterias Gram-negativas se compone de lipopolisacáridos (LPS), porinas y varios sistemas de secreción (Fig. 1.5) (Bos y col., 2007). Los LPS son considerados uno de los constituyentes celulares más variables y desempeñan un importante papel en la evasión bacteriana a los sistemas de defensa del hospedador (Reeves, 1995). El LPS es una molécula anfifílica, que consiste en tres dominios: el lípido A embebido en la membrana, un oligosacárido central y una cadena O distal (Evans y col., 2010). El lípido A posee la actividad endotóxica del LPS, como ha sido demostrado usando lípido A obtenido sintéticamente (Galanos y col., 1995). Dentro de las actividades biológicas más relevantes están la potente activación de macrófagos y la producción de gran cantidad de citoquinas y otros mediadores con múltiples efectos en diferentes órganos (Morrison y Ryan, 1987). La cadena O constituye la porción inmunodominante de la molécula, y los determinantes estructurales de esta región proveen la base de la clasificación serológica de la familia *Enterobacteriaceae* (Davies, 1990). Algunos autores sugieren que la diversidad en estructura y composición de la cadena O pudo haberse desarrollado durante la evolución, con el fin de escapar al sistema inmune del hospedador, mediante la presentación de nuevas especificidades en la superficie celular y así esconder las unidades más profundas, esto es, lípido A y núcleo interno, los cuales son esenciales para el crecimiento y multiplicación bacteriano (Galanos y col., 1995). Asi, la cadena O protegería a las bacterias contra la fagocitosis y contra la acción bactericida del suero.

La variabilidad de perfiles de LPS de *Y. ruckeri* ha permitido establecer relación con los diferentes serotipos (Romalde y col., 1993). De esta manera, las cepas pertenecientes al serotipo O1a (comúnmente asociadas a las epizootias)

presentan el mismo patrón de LPS. Se han descrito los LPS como las moléculas inmunógenicas dominantes en las vacunas para la ERM preparadas con células formolizadas (Amend y Johnson, 1983). La existencia de diferentes estructuras en los LPS de las cepas de serotipos y biotipos emergentes de *Y. ruckeri,* podría explicar la falta de protección inmunológica ante la ERM. Por otra parte, en un estudio reciente se hallaron diferencias entre la estructura de los LPS de cepas móviles e inmóviles del serotipo O1 (Tinsley y col., 2011). Sin embargo estos autores no pudieron establecer ninguna correlación con la virulencia.

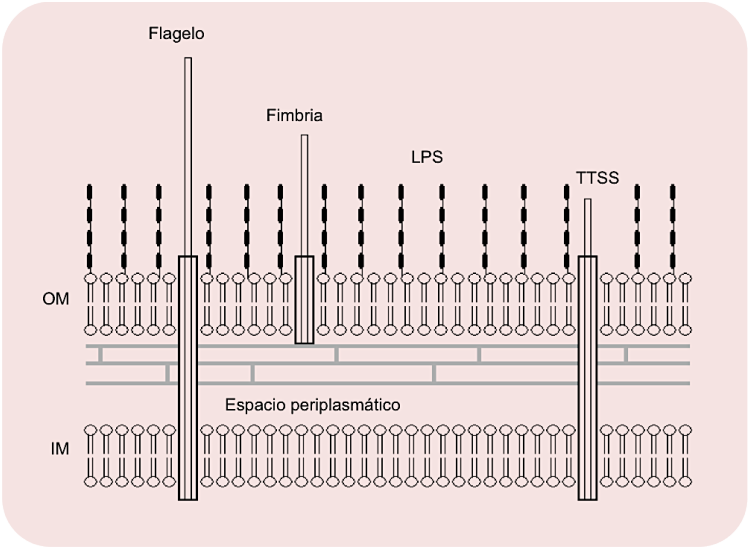

Figura 1.5. Ilustración de la superficie celular de una bacteria Gram-negativa, con sus potenciales principales adhesinas, incluyendo subunidades de flagelos, fimbrias y proteínas de membrana externa. Las adhesinas son generalmente componentes integrales de la membrana externa (OM), pero también se pueden secretar componentes que están asociados con componentes integrales de la membrana. (IM: membrana interna; LPS: lipopolisacáridos; TTSS: Sistemas de secreción). (Obtenido de Tooback, 2009).

Los componentes de la envoltura celular así como pili o fimbrias de las bacterias patógenas son los principales mediadores en la adherencia al hospedador. En esta unión también influyen las propiedades de la célula, es decir, la hidrofobicidad o carga de la superficie celular. Como ya se ha mencionado antes, la adhesión es importante en la colonización del huésped, pero no necesariamente un signo de patogenicidad, ya que generalmente hay múltiples factores implicados en ésta. La hidrofobicidad de la superficie celular se ve influenciada por los polipéptidos y las proteínas (An y Friedman, 2000). Se ha considerado que esta asociación es beneficiosa para el patógeno, ya que permite a las células entrar en contacto con los macrófagos en los que son capaces de multiplicarse. Mantle y Husar (1993) encontraron correlación en la adhesión y la virulencia de *Y. enterolitica* en moco de humanos y conejos. Santos y col. (1991) observaron que las cepas de *Y. ruckeri*, eran no hidrofóbicas, lo que sugirió que otros componentes celulares, tales como LPS, podrían ser importantes en el proceso. Romalde y col. (1990), observaron en general una baja hidrofobicidad en *Y. ruckeri* independientemente del serotipo, además encontraron que se podían observar diferentes grados de hidrofobicidad dependiendo del método utilizado para determinar esta propiedad.

1.4.3.6. Sistemas de secreción

Gunasena y col. (2004) analizaron las secuencias de genes que codifican las proteínas altamente conservadas de los T3SS en *Y. ruckeri* como la ATPasa y los genes adyacentes, y encontraron homología con genes codificantes de aparatos de secreción (Ysa) de los T3SS de *Y. enterocolitica* biovar 1B, sugiriendo la presencia de un Ysa similar en *Y. ruckeri*. El T3SS de *Y. enterocolitica* biovar 1B contribuye en las etapas gastrointestinales de la infección y al suministro de proteínas efectoras, llamadas Ysps, en las células durante la infección (Venecia y

Young, 2005). Varias de las Ysps parecen ser homólogas a otros factores de virulencia, pero se requiere más investigación para entender cómo *Y. enterocolitica* biovar 1B interactúa con las células huésped, así como para revelar la presencia y la función Ysa de los T3SS en *Y. ruckeri*.

Por otra parte, se ha descubierto en *Y. ruckeri* un grupo de ocho genes codificado cromosómicamente, llamado operón *tra*HIJKCLMN, que forman parte de un sistema de secreción tipo IV (T4SS) relacionado con la virulencia (Méndez y col., 2009). Este operón se encontró en cepas de *Y. ruckeri* de diferentes orígenes, lo que indica que estos genes son importantes en la patogénesis de esta bacteria. Los T4SS en patógenos intracelulares participan de la transferencia de diferentes moléculas efectoras dentro de las células hospedadoras, desempeñando un papel importante en la supervivencia dentro de macrófagos o eritrocitos de la sangre. Por lo tanto, el operón *tra* encontrado en *Y. ruckeri* puede ser importante durante la etapa intracelular (Méndez y col., 2009). Al igual que otros factores de virulencia, la expresión de este operón es dependiente de la temperatura siendo sobreexpresado a 18 °C, así como bajo condiciones de limitación de nutrientes. Este operón *tra* es similar al observado en el plásmido de virulencia pADAP de *Serratia entomophila* tanto en secuencia como en organización genética, mientras que no tiene parecido con ningún otro operón *tra* encontrado hasta ahora en yersinias patógenas para humanos.

1.5. Diagnóstico

El diagnóstico rápido de cualquier enfermedad en animales acuáticos es importante para la gestión de los brotes de enfermedades. El aislamiento de *Y. ruckeri* es muy fácil utilizando técnicas clásicas de cultivo en agar tripticasa soja,

agar sangre y agar MacConkey entre otros (ver Tabla 1.2), pudiendo ser rápidamente identificada por sus características bioquímicas. Las principales características bioquímicas que pueden usarse para distinguir positivamente a *Y. ruckeri* de otras especies son: presencia de β-galactosidasa, lisina y ornitina descarboxilasa, no producción de H_2S ni indol, fermentación de glucosa y manitol y no utilización de inositol, ramnosa, sucrosa, melibiosa y arabinosa (Frerichs, 1993; Bottone y col., 2005). Otras características bioquímicas de *Y. ruckeri* como Voges-Proskauer, rojo de metilo, utilización de citrato, hidrólisis de gelatina, hidrólisis del Tween 80 y fermentación de sorbitol, presentan variabilidad entre diferentes aislados (Stevenson y Daly, 1982).

Y. ruckeri puede ser identificada positivamente con el uso de kits comerciales como el sistema API 20E. La variabilidad de las características bioquímicas de *Y. ruckeri,* pueden determinar diferentes perfiles (Tabla 1.7) (Romalde y Toranzo, 1991; Austin y col., 2003; Fouz y col., 2006). Sin embargo, estos deben utilizarse con precaución o como prueba complementaria ya que, algunas cepas producen reacciones falsas que pueden dar lugar a confusiones en la identificación de la bacteria. Los sistemas API ZYM y API 50CH también se han empleado para caracterizar diferentes aislados de *Y. ruckeri* detectándose la producción de las enzimas fosfatasa alcalina, leucina arilamidasa, tripsina, fosfatasa ácida, β-galactosidasa, α-glucosidasa, N-acetil-β-glucoaminidasa, esterasa y esterasa lipasa (Joh y col., 2010), y la utilización de glicerol, D-ribosa, D-galactosa, D-glucosa, D-fructosa, D-manosa, N-acetilglucosamina, D-maltosa, D-trehalosa y en algunas cepas D-sorbitol, como fuentes de carbono (Dear, 1988).

La falta de antisueros ampliamente disponibles contra los diferentes serotipos y biotipos de *Y. ruckeri* hace difícil la identificación serológica para muchos laboratorios. No obstante, el patógeno puede ser identificado por sus

características serológicas, mediante pruebas de aglutinación y otros métodos como inmunoadsorción enzimática (ELISA) o inmunofluorescencia (IFAT) (Smith y col., 1987). Aunque estos métodos inmunológicos han sido útiles en la identificación de *Y. ruckeri*, sólo es posible identificar los aislados/serotipos particulares con los anticuerpos específicos desarrollados debido a la heterogeneidad de los aislados (Romalde y col., 1993). Los anticuerpos monoclonales (Mabs) han ejercido un papel importante en el diagnóstico de muchos patógenos de peces bacterianas (Adams y col., 1995; Wagner y col., 1999). Sin embargo, hasta la fecha muy pocos anticuerpos monoclonales específicos se han desarrollado para la detección o la caracterización de *Y. ruckeri* (Furones y col., 1993).

Las técnicas moleculares como la reacción en cadena de la polimerasa (PCR) (Gibello y col., 1999; Temprano y col., 2001; Altinok y col., 2008) y el análisis de polimorfismos en la longitud de fragmentos de restricción (RFLP) (García y col., 1998) son las más usadas comúnmente, para complementar la rutina de diagnóstico y confirmar la identificación de *Y. ruckeri*, basados en la amplificación selectiva del gen 16S rRNA. Además se ha propuesto otra técnicas de PCR usando los genes *yru*R/*yru*I, responsables de sistemas de "quorum sensing" en *Y. ruckeri*, con alta especificidad (Temprano y col., 2001). La amplificación isotérmica del DNA, LAMP ("Loop-mediated isothermal amplification") basada en estos mismos genes también se ha usado para detectar *Y. ruckeri* en tejidos de peces infectados (Saleh y col., 2008). LAMP tiene una clara ventaja sobre la PCR estándar, ya que es fácil de realizar y requiere solamente un conjunto de cebadores y un baño de agua.

Tabla 1.7. Perfiles API 20E generados por cepas de *Yersinia ruckeri.*

Perfil	Identificación por el índice API
1104100	*Y. ruckeri*
1104501	No codificado
1104700	No codificado
1105100	No codificado
1105500	No codificado
1107100	No codificado
1107500	No codificado
5104100	*Y. ruckeri*
5105100	*Y. ruckeri / Hafnia alvei*
5105500	No codificado
5107100	No codificado
5304100	*Y. ruckeri / H. alvei*
5305100	*Y. ruckeri / H. alvei*
5305500	*Y. ruckeri / H. alvei*
5307100	No codificado

Por otra parte, el rápido avance de las técnicas moleculares, así como la disponibilidad de nuevas secuencias de genes, ha impulsado el desarrollo de técnicas mas sensibles para el diagnóstico de *Y. ruckeri* como la pruebas de aglutinación y otros métodos como inmunoadsorción enzimática (ELISA) o inmunofluorescencia (IFAT). De esta manera, se han propuesto recientemente dos nuevos protocolos para la detección cuantitativa de este patógeno en riñón de peces, basados en los genes 16S rRNA y *gln*A (Glenn y col., 2011; Keeling y col., 2012). La emergencia de las cepas de *Y. ruckeri* del BT2 en peces vacunados, ha resaltado la necesidad de desarrollar técnicas sensibles y rápidas que permitan detectar y diferenciar ambos fenotipos. En un estudio reciente, Welch y col.

(2011) han propuesto un ensayo rápido, basado en una PCR, para la detección de los genotipos de los cuatro alelos mutantes descritos como causantes del fenotipo BT2 en cepas de *Y. ruckeri,* circulantes en Europa y USA. El ensayo se basó en la detección de cambios en los alelos mutantes y en los sitios de corte de las enzimas de restricción en los productos de PCR específicos.

Aunque estas técnicas han permitido la detección de *Y. ruckeri* de un modo rápido y preciso, su utilidad en muestras no letales como la sangre, no fue comprobada. El uso de muestras de sangre no requiere la necropsia y permite muestreo repetidos en peces. Aunque se han descrito dos protocolos de PCR para la detección de *Y. ruckeri* en sangre de trucha arcoíris, ambos estudios mostraron poseer baja sensibilidad (en el procedimiento de extracción de DNA) y/o problemas de inhibición durante el desarrollo de la PCR (Argenton, 1996; Altinok y col., 2001). Otros métodos no letales para el diagnóstico de las infecciones por *Y. ruckeri* se han orientado al cultivo de las heces obtenidas de intestino posterior (Busch y Lingg, 1975; Rodgers, 1992) y de la biopsia de riñón cefálico (Noga y col., 1988).

1.6. Prevención y Control

1.6.1. *Compuestos antimicrobianos*

En la acuicultura, se emplean antibióticos principalmente para impedir (uso profiláctico) y tratar (uso terapéutico) enfermedades bacterianas (Spencer y col., 1997). En el caso de la ERM, frecuentemente se usan agentes antimicrobianos tales como el ácido oxolínico (10 mg/kg pez/día por 10 días), la oxitetraciclina (65 mg/kg pez/día por 10 días) y sulfamerazina (200 mg/kg pez/día por 3 días) para el tratamiento de los brotes de la enfermedad (Rodgers y Austin, 1983; Schmidt y

col., 2000). Los primeros tratamientos con antibióticos se realizaron con sulfamerazina seguida de cloranfenicol u oxitetracilina (Rucker, 1966). Posteriormente, la administración de sulfamidas potenciadas (50 mg/kg pez/día durante 5 días) ha resultado de mucha utilidad en el control tanto de infecciones experimentales como naturales (Bullock y col., 1983; Ledo y col., 1987).

Aunque pueden controlar el problema, el uso de antibióticos causa una serie de desventajas, tales como desarrollo de resistencias bacteriana, recurrencia de nuevos brotes y altos costos (Rodgers, 2001). De esta manera, a pesar que *Y. ruckeri* es sensible a muchos quimioterápicos (Tabla 1.8), se ha descrito resistencia de muchas cepas a la sulfamerazina y oxitetraciclina (De Grandis y Stevenson, 1985; Post, 1987), así como a la tetraciclinas y sulfonamidas (Rigos y Stevenson, 2001) entre otros. Rodgers (2001) estudiaron el incremento de las resistencias en *Y. ruckeri* después de la exposición persistente al ácido oxolínico, la oxitetraciclina y el trimetroprim-sulfametoxazol en condiciones *in vitro*. Los resultados de ese estudio sugieren que la emergencia de cepas de *Y. ruckeri* resistentes a antimicrobianos podría ser minimizada mediante el uso adecuado de los quimioterápicos, optimización de los tratamientos y del ciclo de uso de los agentes quimioterapéuticos disponibles.

Los estudios más recientes se han dedicado a evaluar el uso potencial del ácido caprílico y algunos derivados (monocaprilina y caprilato de sodio) como agentes antimicrobianos contra *Y. ruckeri*. Los resultados han mostrado concentraciones mínimas inhibitorias (CMI) a partir de 7,5 mM para estos compuestos (Kollanoor y col., 2007). Sin embargo, se requieren nuevos estudios para la validación de estos lípidos como antimicrobianos en la acuicultura.

Tabla 1.8. Concentraciones mínimas inhibitorias (CMI) de diferentes antimicrobianos en cepas de *Yersinia ruckeri.* (Rodgers y Austin, 1983)

ANTIMICROBIANO	Rango de CMI (µg/ml)
Acido oxolínico	< 0,5
Acido nalidíxico	≤ 0,5–1,0
Carbenicilina	≤ 0,5–2,0
Cefaloridina	2,0–16,0
Cloranfenicol	2,0–4,0
Eritromicina	16,0–64,0
Estreptomicina	4,0–16,0
Furazolidona	2,0–16,0
Gentamicina	≤ 0,5–2,0
Kanamicina	4,0–8,0
Novobiocina	16,0– ≥ 512,0
Oxitetraciclina	1,0–256,0
Penicilina	16,0–64,0
Polimixina B	1,0– ≥ 512,0
Rifampina	8,0–16,0
Sulfametoxazol	2,0–512,0
Sulfameracina	2,0–512,0
Sulfonamida	0,0156–64,0
Sulfonamida potenciada[a]	0,5:0,25–0,4:2,0
Tetraciclina	1,0–128,0
Tiamulina	16,0–128,0
Trimetoprina	≤ 0,5–1,0
Vancomicina	≥ 512,0

[a] Ormetoprina: Sulfadimetoxina

1.6.2. *Vacunación*

El usos de antibióticos para el control de patógenos bacterianos en la acuicultura conlleva efectos colaterales como acumulación del producto en el músculo del pez, desarrollo de resistencia bacteriana y contaminación de los ambientes acuáticos, sumado a los altos costos de implementación (Heppell y Davis, 2000; Furushita y col., 2003). Una alternativa al uso de estas sustancias ha sido la vacunación, como una de las herramientas más valiosas para prevenir enfermedades en acuicultura.

La yersiniosis fue la primera enfermedad de peces frente a la cual se desarrolló una vacuna, registrándose la primera vacuna comercial en 1976. La vacunación con bacterinas ha sido durante las últimas tres décadas de mucha utilidad en el control eficaz de las infecciones causadas por *Y. ruckeri* en la salmonicultura. No obstante, es sabido que aunque provee un buen nivel de protección, la vacunación no elimina el agente causal, por lo que nuevos brotes de la enfermedad suelen ocurrir a lo largo del tiempo, debidos principalmente a peces portadores bajo condiciones de estrés (Stevenson, 1997).

La ruta por la cual se administra una vacuna puede ejercer un efecto diferente sobre la respuesta inmune. La administración de la vacuna a través de inyección intraperitoneal proporciona los mejores niveles de protección (Palm y col., 1998). La actual estrategia de vacunación contra la ERM involucra un baño o inyección en la etapa de alevines. Debido a la naturaleza de la vacunación por inmersión, la protección es menos duradera con respecto a la vacunación por inyección, por lo que es habitual utilizar un refuerzo de la vacuna vía oral a los 6 meses después de la vacunación (Tatner y Horne, 1985).

Ciertamente, la estrategia de vacunación debería coincidir con registros históricos de la enfermedad en una granja específica, lo que permitiría diferenciar

infecciones endémicas anuales de brotes estacionales (Roberts, 1983). Los brotes estacionales de la enfermedad se asocian con bajas temperaturas del agua en el inicio de los eventos de la temporada y condición de estrés, como la manipulación y clasificación (Roberts, 2001). Debido a que los peces son más susceptibles a la ERM a un tamaño de ~4 g, se recomienda aplicar una vacuna por baño cuando los peces alcanzan esa talla (Hastein y col., 2005). El resultado final es que la vacunación pueda prevenir la enfermedad clínica, pero desafortunadamente no necesariamente eliminará el estado portador en los peces (Bruno y Munro, 1989).

La formulación de la mayoría de las vacunas comerciales contra la yersiniosis está basada sólo en cepas del serotipo O1a/biotipo 1 por ser la causante en la mayoría de los casos de la enfermedad y por lo tanto la más virulenta (Davies, 1990; 1991a; Raida y Buchmann, 2009). Aunque esta vacuna monovalente ofrece diferentes grados de protección cruzada con las demás serovariedades (Cipriano y Rupenthal, 1987; Stevenson, 1997), se han descrito casos de ERM en cultivos de salmónidos previamente vacunados contra la yersiniosis, causadas por cepas que representan nuevas variantes antigénicas (Austin y col., 2003; Fouz y col., 2006; Arias y col., 2007). El desarrollo de autovacunas en algunos de estos casos, ha permitido mantener el control de los nuevos brotes en los sistemas de cultivo. Sin embargo, se requieren estudios en profundidad que permitan obtener nuevas estrategias de protección más eficaces a largo plazo.

Actualmente, existe una vacuna bivalente (RELERA, S. P. Acuicultura) producida contra los biotipos 1 y 2 de *Y. ruckeri*, que fue comercializada en 2008. La vacuna es una preparación formolizada de células enteras tanto del BT1 como del BT2. Hasta la fecha, no hay información acerca de la eficacia de esta vacuna en el campo. Sin embargo, se ha demostrado que esta vacuna confiere protección

ante la inoculación experimental de cultivos virulentos de ambos biotipos (Austin y col., 2003).

En los últimos años, la investigación en el campo de la vacunación se ha centrado en el estudio de nuevas formulaciones utilizando como antígenos proteínas de membrana y lipopolisacáridos (Baba y col., 1988) y la proteasa Yrp1 (Fernández y col., 2003), obteniéndose una alta protección contra la yersiniosis. Las investigaciones más recientes han conseguido mayor eficacia con una vacuna preparada con células vivas atenuadas utilizando una cepa autotrófica mutante de *Y. ruckeri*, en la cual se ha modificado el gen *aro*A (Temprano y col., 2005). Sin embargo, el uso de vacunas con células vivas o con organismos modificados genéticamente requiere cuidadosos estudios debido al riesgo implicado en la liberación, dispersión y supervivencia de estas bacterias en el ambiente natural (Vivas y col., 2004).

1.6.3. *Otras estrategias preventivas*

1.6.3.1. Inmunoestimulantes

La resistencia a patógenos bacterianos puede incrementarse mediante el suministro de inmunoestimulantes, por sí solos o para aumentar la efectividad de la vacuna. En el caso de la yersinosis, los primeros estudios realizados demostraron que la mortalidad causada por *Y. ruckeri* era más baja en peces alimentados con dosis excesivas de vitamina E (Furones y col., 1990).

El β-hidroxi-β-metilbutirato (HMB), un metabolito esencial en la producción de proteínas en los tejidos, cuando es administrado en dosis orales de 50 mg/kg^{-1} durante 8 días después de la inmunización de truchas arcoíris, provee un efecto estimulante contra *Y. ruckeri*. Este compuesto contribuye a la activación de los mecanismos de defensa humoral y celular en el pez y confiere protección

contra las enfermedades (Siwiki y col., 2003). Otros estudios más recientes, sugieren el uso potencial de las proteínas de estrés térmico (HSPs) (del inglés *Heat Shock Protein*) para modular la respuesta inmune innata y adaptativa en peces contra *Y. ruckeri* en condiciones de estrés (Ryckaert y col., 2010).

1.6.3.2. Probióticos

Aunque el control de la yersiniosis está principalmente enfocado en el uso de compuestos antimicrobianos y vacunas, recientemente se ha estudiado la eficiencia de aplicación de probióticos como suplementos alimentarios para estimular y modular la respuesta inmune en los peces contra *Y. ruckeri.* En este sentido, el suministro de esporas de *Bacillus subtilis* y *B. licheniformis* (1:1 4 x 10^4 esporas/g^{-1}) aporta resistencia contra las infecciones de *Y. ruckeri* (Raida y col., 2003). Igualmente, el suplemento de la dieta (> 10^7 cél/g^{-1}) con *Carnobacterium maltaromaticum* o *C. divergens,* así como de *Enterobacter cloacae* y *B. mojavensis* (10^8 cél/g^{-1}), también ofrece protección contra *Y. ruckeri,* aumentando la tasa de supervivencia, la ganancia de peso y mejorando significativamente las constantes hematológicas (Kim y Austin, 2008; Capkin y Altinok, 2009).

Un último estudio en este área ha demostrado además, el uso potencial de componentes subcelulares (LPS, OMPs, WCPs) de los probióticos *Aeromonas sobria* y *B. subtilus* para el control efectivo de *Y. ruckeri* en la trucha arcoíris, incluyendo cepas emergentes del biotipo 2 resistentes a la vacuna (Abbass y col., 2010). Estos resultados parecen indicar el papel importante de estos microorganismos probióticos en el control y prevención de este patógeno, así como también en la disminución del uso de antibióticos en la acuicultura.

1.6.3.3. Bacteriófagos

Los bacteriófagos son útiles en el diagnóstico de laboratorio para la identificación de bacterias patógenas (fagotipado). Sin embargo, se han puesto a punto métodos que permiten evaluar la capacidad de los fagos como agentes terapéuticos en acuicultura (Park y col., 2000; Nakai y Park, 2002).

Un estudio de bacteriófagos contra *Y. ruckeri* permitió detectar y aislar 8 fagos activos (Stevenson y Airdrie, 1984a). De estos, el fago Yer41 causó lisis de todas las cepas estudiadas de la serovariedad I pero fue inactivo contra otras 15 cepas pertenecientes a las otras serovariedades. Otros 6 fagos causaron lisis en cepas de las serovariedades difererentes de la serovariedad I. El fago YerL62 obtenido por inducción con mitomicina C, fue específico para una cepa de la serovariedad V. El patrón de sensibilidad a fagos de *Y. ruckeri* observado en 35 cepas del serotipo I se ha correlacionado con otros factores como patrón de plásmidos o la capacidad de crecimiento a 37 ºC. Por otro lado, estos bacteriófagos, particularmente Yer41, tienen un valor potencial en el diagnóstico de ERM en peces, así como en el estudio de las variaciones entre estas cepas.

Se deben ampliar los conocimientos científicos y optimizar la metodología de que se dispone en la actualidad, para conocer en mayor detalle los límites auténticos del uso de los fagos como agentes terapéuticos, tanto por sí mismos, como complementarios al uso de los antibióticos u otras aproximaciones preventivas de protección frente a las infecciones bacterianas.

1.6.3.4. Resistencia hereditaria

Recientemente se ha detectado que existe una variación genética hereditaria en la resistencia de la trucha arcoíris a *Y. ruckeri*, (Henryon y col., 2005), lo cual plantea un posible enfoque complementario para controlar esta enfermedad mediante la cría selectiva de peces resistentes. Raida y Buchmann (2009),

sugieren que algunos factores de inmunidad innata (citoquinas pro-inflamatorias y proteínas de fase aguda) desempeñan un rol activo durante las infecciones primarias de *Y. ruckeri*, y que algunos mecanismos de inmunidad adaptativos pueden ser activados durante este proceso, siendo posteriormente útiles en la eliminación rápida del patógeno durante periodos de re-infección.

1.7. Re-emergencia de la enfermedad de la boca roja en el cultivo de salmónidos.

Como ya se ha mencionado con anterioridad, las mortalidades debidas a la yersiniosis en los cultivos de salmónidos han sido comúnmente producidas principalmente por cepas pertenecientes al serotipo O1a, biotipo 1. Sin embargo, en los últimos cinco años se han identificado como responsables de las epizootias nuevas cepas de *Y. ruckeri*, para las cuales la vacunación no ha tenido eficacia (Tabla 1.9). La mayoría de estas cepas emergentes de *Y. ruckeri* carecen de movilidad y, mientras en España y USA han sido clasificadas dentro del serotipo O1 biotipo 2 (Fouz y col., 2006; Arias y col., 2007), en Inglaterra se han designado como un nuevo biogrupo que constituye un grupo clonal diferente (Austin y col., 2003). Otros de estos casos descritos en España (con menor incidencia y tasa de mortalidad) han sido causados por cepas móviles del serotipo O2b (Romalde y col., 2003).

Es difícil determinar la razón por la cual las vacunas comerciales no han conferido protección cruzada en estos casos, especialmente porque se desconocen la mayoría de los antígenos presentes en la vacuna. No obstante, algunas proteínas de membrana involucradas con la movilidad, como las flagelinas, son responsables de estimular una respuesta inmune, por lo que, la ausencia de la

misma podría conferir una ventaja selectiva en las cepas inmóviles emergentes (Fouz y col., 2006; Fernández y col., 2007). Por otra parte, la pérdida de movilidad asociada también con la incapacidad de hidrolizar el Tween 80 en el BT2 de *Y. ruckeri*, se ha comenzado a explicar a partir de estudios realizados en la especie afín *Y. enterolitica*, en los que se muestra que la secreción del factor de virulencia fosfolipasa (YplA) depende de un aparato de secreción flagelar competente del tipo III (Young y col., 1999).

En *Y. ruckeri,* mutaciones análogas en genes de secreción flagelar podrían resultar en la pérdida de movilidad, asociada a la falta de secreción de fosfolipasa. Evenhuis y col., (2009) plantean que aunque la falta de movilidad o de actividad fofolipasa pueden afectar a la virulencia en cepas de *Y. ruckeri* del BT1, esta pérdida mutacional puede representar un mecanismo responsable de la emergencia de cepas virulentas de *Y. ruckeri* del BT2.

En Chile, segundo país en importancia en el cultivo de salmones (*S. salar*) a nivel mundial después de Noruega, el uso desde 1995, de la vacuna frente a yersiniosis, ha sido de utilidad para mantener un control exitoso de *Y. ruckeri* (Bravo, 1993). Sin embargo, también han ocurrido varios brotes recientes de yersiniosis, en los que se ha aislado como agente causal, cepas de *Y. ruckeri* del BT1, la mayoría correspondientes con el serotipo O1b de Romalde y col., (1993). Por otra parte, también se han presentado mortalidades en trucha arcoíris cultivadas en Perú (peces no vacunados) causadas por *Y. ruckeri*, de las cuales se han aislados también cepas del BT2. Los últimos casos de brotes de ERM en truchas cultivadas previamente vacunados contra *Y. ruckeri* han tenido lugar durante el año 2008 y 2009, en piscifactorías de Portugal y Finlandia. El estudio sobre la caracterización y relevancia epidemiológica de estos aislados se describen en la presente tesis.

Tabla 1.9. Distribución de cepas emergentes de *Yersinia ruckeri* causantes de nuevos brotes de yersiniosis en cultivos de salmónidos vacunados.

PAÍS	SEROTIPO	BIOTIPO	HOSPEDADOR	REFERENCIA
Inglaterra	O1	2	*O. mykiss*	Austin y col., 2003
España	O2b	1	*O. mykiss*	Romalde y col., 2003
España	O1	2	*O. mykiss*	Fouz y col., 2006
USA	O1b	2	*Salmo trutta*	Arias y col., 2007; Este trabajo
Chile	O1a, O1b, O2b	1	*S. salar*	Este trabajo
Portugal	O1a	1, 2	*O. mykiss*	Este trabajo
Perú[a]	O1a	2	*O. mykiss*	Este trabajo
Australia	O1b	1	*S. salar*	Costa y col., 2011
Finlandia	O1a	2	*O. mykiss*	Ström-Bestor y col., 2011

[a] En el caso de Perú, los peces no habían sido vacunados previamente frente a la ERM. No obstante, estos representan los primeros aislamientos de *Y. ruckeri* causantes de importantes mortalidades en este país.

El BT2 se describió como causante de mortalidades por primera vez, en Inglaterra en la trucha arcoíris (Davies y Frerichs, 1989). En USA, los primeros aislamientos de *Y. ruckeri* biotipo 2, se describieron más recientemente, entre 2002 y 2005, en *S. trutta* (Arias y col., 2007). Estos estudios plantean que la diseminación de estos aislados es compleja. Wheeler y col. (2009) sugieren que el biotipo 2 ha podido emerger independientemente en Europa continental e Inglaterra representando grupos clonales diferentes, aún cuando ha existido intercambio de aislados desde USA en el pasado.

No obstante, es relevante la necesidad de nuevos estudios que permitan dilucidar las posibles causas de diseminación y diversificación de este importante

patógeno, así como también las interrelaciones filogenéticas entre estos aislados y sus aspectos poblacionales. Esta información resultaría útil para el entendimiento de su dinámica evolutiva y desarrollo de estrategias más eficaces de control y prevención.

1.8. Enfoque evolutivo

El análisis filogenético ha jugado un papel central en la microbiología básica. Los datos de secuencia genealógica ofrecen información directa que se puede utilizar de manera eficiente para estimar las relaciones filogenéticas y los parámetros asociados a la dinámica poblacional. (Feil y col., 2004; Spratt y col., 2004). La reconstrucción de los patrones de descendencia para un grupo de organismos permiten inferir el origen de los miembros de un grupo con características específicas, y cómo esos organismos se distribuyen en todo el entorno (Lemey y col., 2009). Por otra parte, la integración de estos patrones con conocimientos de su filogenia proporciona información detallada sobre el seguimiento epidemiológico de un organismo a diferentes escalas evolutivas, desde un único origen y a través del mundo (Moodley y col., 2009).

Para los patógenos microbianos, los análisis filogenéticos se realizan a menudo con el fin de determinar si un brote en particular puede estar relacionado con otros durante los tiempos de una epidemia. Si bien la naturaleza clonal de un brote puede ser fácilmente medida y predicha, Maynard y col. (1993) señalaron la importancia potencial de recombinación homóloga como un factor determinante en la estructura de la población general de muchas especies bacterianas. Estas nociones están ahora apoyadas por varios resultados obtenidos gracias a métodos de tipado como MLST. Los niveles de diversidad genética son suficientemente altos en la mayoría de los taxones microbianos, de modo que las secuencias de

varios fragmentos de genes esenciales puede proporcionar una visión general de resolución media de su estructura genética de la población (Maiden, 2006). En el caso de bacterias patógenas, cuyos miembros presentan diversos grados de virulencia, la integración de la genética de poblaciones y evolutiva, así como los estudios epidemiológicos pueden proporcionar importantes conocimientos sobre los orígenes y la propagación de la enfermedad.

La mayoría de las especies muestra una estructura de la población que puede ser interpretada en su contexto geográfico y cronológico. La filogeografía utiliza la información genética para estudiar la distribución geográfica de los linajes genealógicos, especialmente los que se encuentran dentro de las especies (Avise, 2000). Debido a que ésta disciplina tiene raíces profundas en la biogeografía histórica y genética de poblaciones, la filogeografía se percibe como un puente que une el estudio de los procesos micro- y macro-evolutivos que proporcionan el vínculo empírico y conceptual entre la sistemática y la genética de poblaciones. Sobre la base de un muestreo apropiado de individuos y genes, este enfoque permite la evaluación de la descripción de la evolución de unidades de población, y la inferencia de los procesos que subyacen al origen, la distribución y el mantenimiento de la diversidad (Beheregaray y Caccone, 2007).

Así, la generación de grandes volúmenes de datos de secuencias, en combinación con el desarrollo de nuevas técnicas analíticas y los avances conceptuales, prometen una mejor comprensión de la complejidad de la evolución de las poblaciones de bacterias.

2. OBJETIVOS

2. Objetivos:

Es evidente que existe una necesidad de examinar los aspectos filogenéticos y evolutivos de *Y. ruckeri,* en la búsqueda de una mayor comprensión de la epidemiología y persistencia a lo largo del tiempo de este patógeno en el ámbito mundial de la piscicultura. Además, el conocimiento de estas interrelaciones genéticas facilitaran las decisiones para programas de manejo y control sanitario. Debido a que los conocimientos existentes sobre estos aspectos en *Y. ruckeri* son prácticamente inexistentes, los objetivos planteados en esta memoria fueron los siguientes:

1. Caracterizar mediante técnicas bioquímicas, serológicas y moleculares diferentes cepas de *Y. ruckeri,* aisladas de brotes recientes de ERM cultivados en USA, Europa y Suramérica, y evaluar la eficacia del uso combinado de diferentes técnicas de tipado para la discriminación epidémica entre diferentes aislados.
2. Determinar la estructura poblacional de *Y. ruckeri* basado en el desarrollo de un esquema de análisis de tipado de secuencias multilócicas (MLST) e investigar los procesos demográficos que definen la población actual y la filogeografía de este patógeno.
3. Reconstruir la divergencia y transmisión de *Y. ruckeri* a través del tiempo y el espacio, y deducir la ubicación geográfica de cepas ancestrales implementando un enfoque de análisis bayesiano.
4. Desarrollar un protocolo de detección y cuantificación no destructivo mediante qPCR en tiempo real para determinar la distribución de esta bacteria en tejidos de peces.

3. CAPÍTULO I:

CARACTERIZACIÓN FENOTÍPICA, SEROLÓGICA Y MOLECULAR DE CEPAS DE *Yersinia ruckeri* AISLADAS DE RECIENTES BROTES EN AMÉRICA Y EUROPA

Artículo:

Serological and molecular heterogeneity among *Yersinia ruckeri* strains isolated from farmed Atlantic salmon *Salmo salar* in Chile

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Resumen:

Se investigaron 11 cepas de *Yersinia ruckeri*, el agente causal de la enfermedad entérica la boca roja (ERM), aisladas de salmón del Atlántico *Salmo salar* L. cultivados en Chile, y previamente vacunados contra la ERM. El análisis filogenético de las secuencias del gen 16S rRNA confirmó la identificación de los aislados de salmón como *Y. ruckeri*. Se realizó un análisis comparativo de las características bioquímicas utilizando métodos tradicionales y sistemas comerciales miniaturizados. Todas las cepas estudiadas fueron móviles y Tween 80 positivas, siendo identificadas como del biotipo 1. Además, las pruebas de susceptibilidad a fármacos determinaron que todos los aislados mostraron alta sensibilidad al sulfametoxazol/trimetoprima, oxitetraciclina, ampicilina y enrofloxacina. Los ensayos serológicos evidenciaron la presencia de los serotipos

O1a, O1b y O2b, con predominio del serotipo O1b en 9 cepas. El análisis de los perfiles de lipopolisacáridos y el correspondiente inmunoblot confirmaron estos resultados. La electroforesis en geles de poliacrilamida (SDS-PAGE) de las proteínas de membrana externa reveló que todas las cepas chilenas poseían perfiles con un rango de peso molecular entre 34 y 55 kDa, con 3 grupos distintos basados en las diferencias de las bandas principales. Los análisis de secuencias consenso intergénicas repetitivas (ERIC-PCR) y de elementos palindrómicos extragénicos repetitivos (REP-PCR), indicaron claramente la diversidad genética intraespecífica entre las cepas chilenas de *Y. ruckeri*.

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Serological and molecular heterogeneity among *Yersinia ruckeri* strains isolated from farmed Atlantic salmon *Salmo salar* in Chile

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ABSTRACT: We investigated 11 strains of *Yersinia ruckeri*, the causative agent of enteric redmouth disease (ERM), that had been isolated from Atlantic salmon *Salmo salar* L. farmed in Chile and previously vaccinated against ERM. Phylogenetic analysis of the 16S rRNA gene sequences confirmed the identification of the salmon isolates as *Y. ruckeri*. A comparative analysis of the biochemical characteristics was made by means of traditional and commercial miniaturised methods. All studied isolates were motile and Tween 80 positive, and were identified as biotype 1. In addition, drug susceptibility tests determined high sensitivity to sulphamethoxazole/trimethroprim, oxytetracycline, ampicillin and enrofloxacin in all isolates. Serological assays showed the presence of O1a, O1b and O2b serotypes, with a predominance of the O1b serotype in 9 strains. Analysis of the lipopolysaccharide profiles and the correspondent immunoblot confirmed these results. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the outer membrane proteins revealed that all Chilean strains had profiles with a molecular weight range between 34 and 55 kDa, with 3 distinct groups based on differences in the major bands. Genotyping analyses by enterobacterial repetitive intergenic consensus (ERIC-) and repetitive extragenic palindromic (REP-)PCR techniques clearly indicated intraspecific genetic diversity among Chilean *Y. ruckeri* strains.

KEY WORDS: *Yersinia ruckeri* · Enteric redmouth disease · ERM · Atlantic salmon · Serology · Outer membrane proteins · Lipopolysaccharide · Genotyping

INTRODUCTION

Yersinia ruckeri is the causative agent of enteric redmouth (ERM) disease or yersinosis in salmonid and non-salmonid fish reared in both fresh and marine waters. Infection may result in the development of chronic or acute septicaemia with haemorrhages on the body surface and in the internal organs, and high mortalities might occur, particularly in rainbow trout *Oncorhynchus mykiss* (Walbaum) and Atlantic salmon *Salmo salar* (L.). *Y. ruckeri*, which was initially isolated from rainbow trout in the Hagerman Valley of Idaho, USA, in the 1950s (Rucker 1966), is now widely found in fish populations throughout North and South America, Australia, Africa and Europe (Austin & Austin 2007). The apparent spread of the disease throughout the different areas has been associated with the absence of strict controls and appropriate preventive programs (Horne & Barnes 1999).

Yersinia ruckeri is a serologically variable, highly clonal species. The pathogen includes 2 biotypes. Biotype 1 strains are positive for motility and lipase activity, whereas biotype 2 strains are negative for both tests (Davies & Frerichs 1989). The species has been grouped into 6 serovars (Stevenson & Airdrie 1984), 5 O-serotypes (Davies 1990) or 4 O-serotypes with different subgroups (Romalde et al. 1993) by using different serotyping systems. In addition, *Y. ruckeri* strains

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can be grouped into clonal types on the basis of biotype, serotype and outer membrane protein (OMP) profiles (Davies 1991). Strains of serotypes O1a (classic serovar I) and O2b (classic serovar II) cause most epizootic outbreaks, and serotype O1a is predominant in cultured salmonids (Stevenson & Airdrie 1984, Romalde et al. 1993).

ERM has been successfully controlled for decades by vaccination with monovalent killed whole cell commercial vaccines. Although formulations of most commercial vaccines are based only on serovar I (Hagerman strain), different degrees of cross-protection among serotypes have been reported (Stevenson & Airdrie 1984). In recent years, reports of ERM vaccine breakdown have emerged in Europe and the USA, which were mostly attributed to biotype 2 strains (Austin et al. 2003, Fouz et al. 2006, Arias et al. 2007, Wheeler et al. 2009).

In Northern Europe and Chile, both of which are major production areas of farmed Atlantic salmon, the fry are routinely vaccinated with commercial rainbow trout ERM vaccines (Bravo & Midtlyng 2007, Wheeler et al. 2009). In Chile, yersiniosis vaccines came into use in 1995 following the first occurrence of ERM in Atlantic salmon in 1992 (Toledo et al. 1993). In Chile alone, up to 140 million salmon yr^{-1} were vaccinated against *Yersinia ruckeri* between 1999 and 2003. According to the diagnostic laboratories, these vaccines have proven to be efficacious (Bravo & Midtlyng 2007). However, during 2008 some Chilean Atlantic salmon hatcheries suffered a series of mortality episodes by ERM affecting fish previously immunised against *Y. ruckeri*.

In this study we report the full phenotypic, serological and molecular characterisation of a group of *Yersinia ruckeri* strains that were isolated from these ERM epizootics in Atlantic salmon farmed in Chile.

MATERIALS AND METHODS

Bacterial isolates. Several episodes of ERM with mortality up to 10% occurred in different Atlantic salmon farms in Chile during 2008. In all cases, fish had been previously immunised with an autologous vaccine based on strains of *Yersinia ruckeri* of both serotype O1 (Hagerman type strain) and O2b (O'Leary type strain). Diseased fish with average weights from 2 to 60 g showed typical signs of ERM, such as severe haemorrhages in the mouth and eyes, and petechiae in abdominal musculature, liver, visceral fat and pyloric caeca. Bacterial isolates were aseptically collected from internal organs (liver, spleen, kidney, brain and gills). Isolates were routinely cultured and purified on tryptic soy agar (Difco Laboratories) and were kept frozen at −80°C in tryptic soy broth (Difco) supplemented with 15% glycerol. The type strain of the species and representative strains of the different O-serotypes following the scheme of Romalde et al. (1993) were also included in all analyses for comparative purposes (Table 1).

Biochemical and physiological characterisation. Bacterial isolates were biochemically characterised by conventional plate and tube tests by the procedures of Romalde et al. (1993). In addition, all cultures were identified using API 20E and API ZYM (BioMerieux) using saline solution (NaCl 0.85%) for the bacterial suspensions. The study of acid production from carbon sources was tested with API 50CH employing 50 CHB/E medium following the manufacturer's instructions. Strips were incubated at 25°C, and readings were performed from 24 to 48 h (Romalde & Toranzo 1991).

Table 1. *Yersinia ruckeri*. Strains used in this study. ^{T}: type strain. Collections are: NCIMB, National Collection of Industrial, Marine, and Food Bacteria; D. P. Anderson, National Fish Health Research Laboratory, Kearneysville, West Virginia; T. Håstein, National Veterinary Institute, Oslo; R. M. W. Stevenson, University of Guelph, Ontario; T. Cook, University of Maryland

Reference no. (strain)	Source	Collection
Reference strains		
NCIMB 1316 (O1a)	*Oncorhynchus mykiss* (USA)	NCIMB
NCIMB 2194^{T} (O1a)	*O. mykiss* (USA)	NCIMB
O-serotype strains (Romalde et al. 1993)		
11.4 (O1a)	*O. mykiss* (Norway)	D. P. Anderson
1533 (O1b)	*Salmo salar* (Canada)	T. Håstein
RS6 (O2a)	*Salvelinus fontinalis* (USA)	R. M. W. Stevenson
11.29 (O2b)	*O. tshawytcha* (USA)	D. P. Anderson
RS2 (O2c)	*O. mykiss* (USA)	R. M. W. Stevenson
11.47 (O3)	*O. mykiss* (USA)	T. Cook
11.73 (O4)	*O. mykiss* (USA)	T. Cook
Fresh isolates		
2550	*Salmo salar* (Chile, 2008)	Laboratory collection
2576	*S. salar* (Chile, 2008)	Laboratory collection
2599	*S. salar* (Chile, 2008)	Laboratory collection
6807	*S. salar* (Chile, 2008)	Laboratory collection
8526	*S. salar* (Chile, 2008)	Laboratory collection
8386	*S. salar* (Chile, 2008)	Laboratory collection
8930	*S. salar* (Chile, 2008)	Laboratory collection
8958	*S. salar* (Chile, 2008)	Laboratory collection
8959	*S. salar* (Chile, 2008)	Laboratory collection
8960	*S. salar* (Chile, 2008)	Laboratory collection
9394	*S. salar* (Chile, 2008)	Laboratory collection

The antimicrobial susceptibility of the isolates was determined by the disc diffusion method on Mueller-Hinton agar (Oxoid) following the recommendations of CLSI (2009). The following chemotherapeutic agents (µg disc^{-1}; Oxoid) were used: sulphamethoxazole/trimethoprim (25), tetracycline (30), oxytetracycline (30), ampicillin (10) and enrofloxacin (5). In parallel, the minimum inhibitory concentration (MIC) ranges of these drugs were determined using the commercial technique E-test (AB Biodisk). Since oxytetracycline is not available in this commercial test, tetracycline was employed in the E-test assays. Susceptibility to different polymyxin B concentrations (0.9 to 500 µg ml^{-1}) was also evaluated by the disk diffusion method. In all cases, the culture density was adjusted to produce a turbidity level equivalent to that of 0.5 McFarland standard (~1 × 10^8 colony-forming units ml^{-1}). Inhibition zone readings were performed after 48 h of incubation at 25°C.

Serological characterisation. For immunological analysis, antisera raised against the reference strains of different serotypes of *Yersinia ruckeri* were obtained in rabbits as previously described (Romalde et al. 1993). Slide agglutination assays were carried out using whole cells and O-antigens (obtained by heating a cell suspension for 1 h at 100°C) as described by Toranzo et al. (1987). Serological relations were further evaluated by dot blot analysis following the procedures of Cipriano et al. (1985) modified for use with a vacuum system (SNAP, Millipore) according to the manufacturer's instructions. A reaction similar to that shown by the homologous strain was recorded as positive. For these analyses, the different antisera used were previously absorbed with the heterologous strains following the procedures described by Stevenson & Airdrie (1984). Briefly, 500 µl of each serum were subjected to 3 absorption steps (2 h at 37°C) with the heterologous strains (final concentration of 10^9 bacteria ml^{-1}), followed by an overnight incubation at 4°C. After each incubation, bacterial cells were eliminated from the serum by centrifugation (5000 × *g*, 2 min). Absorbed sera were maintained at –20°C until use.

Lipopolysaccharide and membrane protein analysis. The preparation of lipopolysaccharide (LPS) and total and outer membrane proteins (OMP) were performed as previously described (Romalde et al. 1993). Samples were examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970) using 12% acrylamide in the resolving gel and 4% acrylamide in the stacking gel. After electrophoresis, LPS samples were silver stained following the method of Tsai & Frasch (1982), and protein samples were stained with 0.05% Coomassie blue R (Sigma) in 25% propan-2-ol-10% acetic acid. Immunoblotting of LPS components and membrane proteins was done by transferring the components separated by SDS-PAGE to nitrocellulose sheets by electrophoretic blotting following the method of Towbin et al. (1979) and treating as previously described (Romalde et al. 1993). Immunological analysis was carried out employing absorbed antisera as mentioned in 'Serological characterisation'.

16S rRNA gene analysis. Total bacterial DNA was extracted from pure bacterial cultures using the InstaGene matrix (Bio-Rad) following the manufacturer's instructions. The DNA concentration was spectrophotometrically quantified and adjusted to a concentration of 100 ng µl^{-1}. Purified DNA was maintained at –20°C until use. 16S rRNA gene was amplified in a temperature-gradient thermal cycler (Biometra) using the Ready-To-Go PCR beads kit (Pharmacia Biotech), and sequenced in an Automatic DNA sequencer (model 373A, Applied Biosystems) as previously described by Osorio et al. (1999). Additional sequencing was performed for type strains NCIMB 2194^T and NCIMB 1316 of *Yersinia ruckeri*. Sequence data analysis was performed with the DNAstar Seqman program (Lasergene). Sequences of phylogenetically related species were obtained after BLAST (www.ncbi.nlm.nih.gov/blast/) and EzTaxon (EzTaxon server 2.1, http://147.47.212.35:8080/) searches against the latest GenBank releases. Phylogenetic trees were constructed by neighbour-joining (NJ; Saitou & Nei 1987), distance matrices were calculated using Kimura's 2-parameter correction and stability of groupings, and bootstrap analysis (1000 replicates) was conducted using MEGA version 4.0 (Tamura et al. 2007), using *Hafnia alvei* ATCC 13337^T (accession no. M59155) as the outgroup. 16S rRNA gene sequences of the Chilean isolates of *Y. ruckeri* were deposited in the GenBank database under accession numbers FN668381 to FN668391.

REP and ERIC-PCR typing. Repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) sequences from all *Yersinia ruckeri* isolates were analysed as previously described (Versalovic et al. 1991). All amplifications were carried out in a temperature gradient thermal cycler (Biometra), and the PCR products were electrophoresed in agarose gels (1.5% w/v) with Tris-acetate-EDTA (0.004 M Tris-actate, 0.0001M EDTA, pH 8.0) electrophoresis buffer and stained with ethidium bromide (2 µg ml^{-1}). A 50 to 2000 base pair (bp) ladder (Sigma) was used as a molecular mass marker. All gels were scanned, and images were captured by a Gel Doc-2000 gel documentation system (Bio-Rad). Patterns were analysed using the Diversity Database software (Bio-Rad). The computed similarities among isolates were estimated by means of the Dice coefficient (S_d; Dice 1945). Dendrograms were obtained using the unweighted pair group method average (UPGMA).

RESULTS

Biochemical and physiological characterisation

The 11 Chilean isolates studied, which were obtained from different farms and outbreaks, exhibited great phenotypic homogeneity. All strains were Gram-negative fermentative rods, oxidase negative and positive for lysine and ornithine decarboxylase, gelatin and the Voges-Proskauer reaction, which allowed their presumptive identification as *Yersinia ruckeri*. Moreover, all isolates were identified as biotype 1 because of their motility and capacity to hydrolyse Tween 80. The main differential traits among the Chilean isolates and the reference and serotype representative strains used in this study were fermentation of sorbitol, Voges-Proskauer reaction, hydrolysis of gelatin and utilisation of citrate (Table 2). In the API 20E test, 9 Chilean isolates showed the same numeric profile (5107100), while 2 isolates showed minor differences, rendering profiles 5307100 and 5107500. These profiles differed from the profiles shown by the type strain NCIMB 2194^T, serotype representative strains 11.4 (O1a) and 1533 (O1b) (5104100), and the profile detected for RS2 (O2a), 11.29 (O2b), RS6 (O2c) and 11.47 (O3) serotype representative strains (5105500). Again, the biochemical differences were observed in Voges-Proskauer, gelatin hydrolysis, utilisation of citrate and sorbitol fermentation.

Results obtained with the miniaturised test API ZYM indicated positive activity for alkaline phosphatase, leucine arylamidase, trypsine, acid phosphatase, β-galactosidase, α-glucosidase and N-acetyl-β-glucosaminidase in all isolates studied, including reference and serotype representative strains. Moreover, the capacity to ferment glycerol, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, D-acetylglucosamine, D-maltose and D-trehalose was detected in all strains by the API 50 CH test. Only the Chilean isolate 6807 and the serotype representative strains RS2 (O2a), 11.29 (O2b), RS6 (O2c) and 11.47 (O3), were also able to ferment sorbitol.

All Chilean isolates showed a drug-susceptibility pattern similar to that of the reference strains NCIMB 2194^T and NCIMB 1316, being highly susceptible to sulphamethoxazole/trimethoprim, tetracycline, oxytetracycline, ampicillin and enrofloxacin. No differences were observed between standard and E-test methods to determine the sensitivity of the *Yersinia ruckeri* strains studied. Moreover, Chilean isolates were sensitive to polymyxin B with a MIC of 31.2 µg ml^{-1}, while the reference and serotype representative strains showed higher susceptibility (MIC = 15.6 µg ml^{-1}).

Table 2. *Yersinia ruckeri*. Differential biochemical characteristics observed for the strains analysed. ^{T}: type strain; +: positive reaction; –: negative reaction; (n): number of positive strains

Test	Voges-Proskauer	Gelatin	Citrate	Sorbitol
Chilean isolates	+	+	+ (2)[a]	+ (1)[b]
Reference strains				
NCMB 2194^T	–	+	–	–
NCMB 1316	–	+	–	–
O-serotype strains				
11.4(O1a)	–	–	–	–
1533(O1b)	–	–	+	–
RS2(O2a)	+	–	+	+
11.29(O2b)	–	+	+	+
RS6(O2c)	+	+	–	+
11.47(O3)	+	+	+	+
11.73(O4)	–	–	–	–

[a]The 2 positive isolates for citrate utilisation were 8958 and 8959
[b]The positive isolate for acid production from sorbitol was 6807

Serological characterisation

Cross-reactions were observed for all isolates, using whole cells or thermostable O-antigens, when no absorbed antisera were employed (data not shown). Agglutination assays performed using the O-antigens and absorbed antisera revealed the presence of 3 different O-serotypes among the Chilean *Yersinia ruckeri* isolates examined in this study (Table 3). One isolate (2576) was identified as belonging to serotype O1 subgroup a, 9 isolates belonged to serotype O1 subgroup b, and 1 Chilean isolate (6807) reacted only with antisera against the serotype O2 subgroup b reference strain. The results of the dot blot test using absorbed sera support those of slide agglutination.

LPS and membrane protein patterns

Three different profiles were observed in the LPS of the Chilean *Yersinia ruckeri* isolates, similar to those shown by the representative strains of serotypes O1a (1 isolate), O1b (9 isolates) and O2b (1 isolate). In the patterns exhibited by the serotype O1a and O1b isolates, a minor number of bands with a greater distance among them were observed. Immunoblot assays, using absorbed antisera raised against serotype O1a, O1b and O2b, supported these results (Fig. 1).

The SDS-PAGE analysis of the OMPs revealed that the isolates yielded 3 different profiles, which could be related to their serotype. One profile was observed in the reference strain NCIMB 2194^T and in serotype O1a

Table 3. *Yersinia ruckeri*. Serological results obtained for slide agglutination, dot blot and Western blot analysis of Chilean bacterial isolates using antisera of *Y. ruckeri* absorbed with heterologous strains. O1a, O1b, O2b: antisera raised against representative O-serotype *Y. ruckeri* strains 11.4, 1533 and 11.29, respectively (Romalde et al. 1993). ^{T}: type strain; LPS: lipopolysaccharide; OMP: outer membrane protein; +: positive reaction; –: negative reaction

Isolate	Slide agglutination (O antigen)			Dot blot (O antigen)			Western blot (LPS)			Western blot (OMPs)		
	O1a	O1b	O2b	O1a	O1b	O2b	O1a	O1b	O2b	O1a	O1b	O2b
2550	–	+	–	–	+	–	–	+	–	–	+	–
2576	+	–	–	+	–	–	+	–	–	+	–	–
2599	–	+	–	–	+	–	–	+	–	–	+	–
6807	–	–	+	–	–	+	–	–	+	–	–	+
8526	–	+	–	–	+	–	–	+	–	–	+	–
8386	–	+	–	–	+	–	–	+	–	–	+	–
8930	–	+	–	–	+	–	–	+	–	–	+	–
8958	–	+	–	–	+	–	–	+	–	–	+	–
8959	–	+	–	–	+	–	–	+	–	–	+	–
8960	–	+	–	–	+	–	–	+	–	–	+	–
9394	–	+	–	–	+	–	–	+	–	–	+	–
Reference strains												
NCIMB 2194^{T}	+	–	–	+	–	–	+	–	–	+	–	–
NCIMB 1316	+	–	–	+	–	–	+	–	–	+	–	–
11.4	+	–	–	+	–	–	+	–	–	+	–	–
1533	–	+	–	–	+	–	–	+	–	–	+	–
11.29	–	–	+	–	–	+	–	–	+	–	–	+

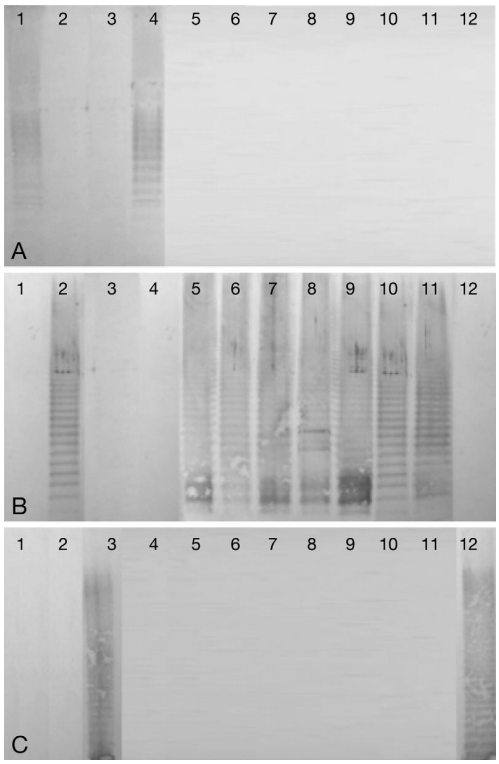

Fig. 1. *Yersinia ruckeri*. Immunoblot assays of the lipopolysaccharide extracted from Chilean isolates using absorbed antisera raised against (A) serotype O1a (11.4), (B) serotype O1b (1533) and (C) serotype O2b (11.29) representative strains. Lanes: 1, 11.4; 2, 1533; 3, 11.29; 4, 2576; 5, 2550; 6, 2599; 7, 8526; 8, 8930; 9, 8959; 10, 8960; 11, 9394; 12, 6807

representative strain 11.4, as well as in the Chilean isolate 2576 (Fig. 2). A second OMP profile included the serotype O1b representative strain 1533 and the Chilean isolates identified as serotype O1b (Fig. 2). The representative serotype O2b strain 11.29 and the Chilean isolate 6807 yielded a third profile (Fig. 2). The Western blot assay of total and OMPs showed correspondence with the dot blot and LPS results, only when absorbed sera were used for the immunological reactions (data not shown).

Genetic characterisation

The partial 16S rRNA gene sequences of all Chilean isolates showed the highest similarity (>99.82%) with *Yersinia ruckeri* NCIMB 2194^{T} (EF179132), followed by *Y. kristensenii* ATCC 33638^{T} (ACCA01000078; 98.45%) and the remaining *Yersinia* species (<98%). Phylogenetic analysis grouped all Chilean isolates with the *Y. ruckeri* type strain, with bootstrap values of 100% (data not shown).

All Chilean *Yersinia ruckeri* strains were typeable, rendering discernible amplification patterns by any of

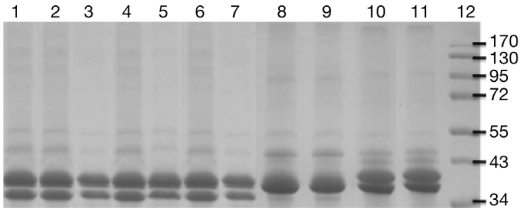

Fig. 2. *Yersinia ruckeri*. Outer membrane protein profiles of the strains studied. Lanes: 1, 1533 (O1b); 2, 8960; 3, 8526; 4, 9394; 5, 2550; 6, 8386; 7, 8958; 8, 11.29 (O2b); 9, 6807; 10, 11.4 (O1a); 11, 2576; 12, molecular size marker. Numbers on the right indicate the molecular size of the markers in kDa

the PCR-based methods used. The banding patterns obtained from ERIC-PCR fingerprints showed profiles with common bands (between 8 and 10) with molecular weights from 50 to 1500 bp. Three different genetic groups were differentiated, which corresponded with the serotypes of the isolates (Fig. 3A). Cluster I grouped reference strains NCMB 1316 and serotype representative strains 11.4 (O1a) and 11.47 (O3). Cluster II included the Chilean isolate 6807 and serotype representative strains RS2 (O2a), 11.29 (O2b) and RS6 (O2c). Finally, Cluster III grouped the rest of the Chilean isolates with the reference strains NCIMB 2194^T and the serotype representative strain 1533 (O1b). Isolates within each group showed homology in their profiles between 88 and 97%. An S_d of 73% was obtained between Clusters II and III, while Cluster I joined them at 66% similarity.

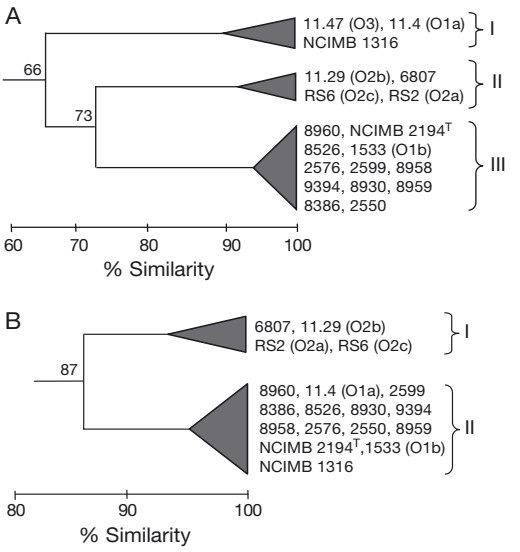

Fig. 3. *Yersinia ruckeri*. Clusters obtained using the Dice similarity coefficient and unweighted pair group method average (UPGMA) analysis based on (A) enterobacterial repetitive intergenic consensus (ERIC)-PCR and (B) repetitive extragenic palindromic (REP)-PCR produced patterns in *Y. ruckeri* strains

Although the band profiles obtained by REP-PCR appeared to be more homogeneous, 2 patterns were detected with 9 and 11 amplification bands ranging from 150 to 2200 bp (Fig. 3B). Cluster I grouped serotype O2 representative strains, RS2 (O2a), 11.29 (O2b) and RS6 (O2c), and also included the Chilean serotype O2b isolate (6807). Cluster II comprised the remaining Chilean isolates, the reference strain NCIMB 2194^T and the serotype O1a and O1b representative strains. Within each genetic group, the strains were highly homogeneous (S_d = 95–100%), and the similarity between Clusters I and II was 87%.

DISCUSSION

Diagnosis of the causative agent of a fish disease is important to implement appropriate control strategies. Outbreaks of ERM occurred in 2008 in Chilean Atlantic salmon that had previously been vaccinated. Samples of these fish were analysed in the current study to confirm the aetiology of *Yersinia ruckeri* and to identify the main phenotypic and molecular characteristics of the responsible isolate.

The biochemical results showed strong homogeneity in the Chilean isolates, all of which belonged to biotype 1 (motile and lipase positive). The only variable biochemical characteristics among them were in the use of citrate and the fermentation of sorbitol. On the other hand, all Chilean isolates showed some biochemical differences to the reference strains NCMB 2194^T and NCMB 1316, which were Voges-Proskauer and gelatinase positive. This variation in some biochemical tests for *Yersinia ruckeri* strains has been reported in other studies (Austin & Austin 2007). Some studies reported the ability to ferment sorbitol as a distinguishing characteristic for strains of *Y. ruckeri* belonging to serotype O2 (Romalde et al. 2003, Austin & Austin 2007). However, some isolates, including the different serotypes O1, O3 and O4, are also able to ferment sorbitol (Stevenson & Airdrie 1984, Sousa et al. 2001). In this study, 1 Chilean isolate (6807) was able to ferment sorbitol and was confirmed by serological tests as belonging to serotype O2b.

The biochemical profiles obtained in the API 20E (5017100, 5307100 and 5107500), although not included in the database API system, have been previously reported for *Yersinia ruckeri* by other authors (Romalde & Toranzo 1991, Austin et al. 2003). The results obtained using the miniaturised systems API

ZYM and API 50 CH were completely homogeneous among all isolates studied and also consistent with those obtained in conventional tests.

Although ERM mortalities in cultured salmonids have been mainly caused by strains of serotype O1a, biotype 1 (Austin & Austin 2007), new strains of *Yersinia ruckeri* have been reported as causal agents in outbreaks affecting salmonids vaccinated against ERM in different geographic areas. Some of these outbreaks were attributed to emergent non-motile, Tween 80 negative, biotype 2 isolates (Austin et al. 2003, Fouz et al. 2006, Arias et al. 2007). Other cases (with lower incidence and mortality) were caused by strains of serotype O2b (Romalde et al. 2003).

The few reports available on *Yersinia ruckeri* in Chile described serotype O1a strains as the primary cause of outbreaks, with serotype O2 strains only occasionally detected (Troncoso et al. 1994). In contrast, the predominant serotype that we found in Chilean strains was serotype O1b (former serovar III), suggesting that commercial vaccines do not protect against this variant. The different LPS patterns exhibited by the Chilean isolates corresponded to those of serotypes O1a, O1b and O2b. Differences in the number of bands and the interband distances among serotypes are consistent with results previously reported for *Y. ruckeri* by other authors (Romalde et al. 1993, Sousa et al. 2001). Results of OMP analysis of the Chilean *Y. ruckeri* isolates supported those from previous studies (Romalde et al. 1993, Sousa et al. 2001), in which observations of distinct protein profiles were observed related to the different serotypes of *Y. ruckeri*.

In addition, genetic variability was observed in Chilean *Yersinia ruckeri* isolates using REP- and ERIC-PCR techniques, establishing, respectively, 2 and 3 groups related with the serotype. ERIC-PCR showed higher discriminatory power than REP-PCR, but both techniques could have some value as tools to study ERM epidemiology.

Finally, the facts that Chilean isolates in this study were associated with mortalities by ERM in salmon previously vaccinated and that they belonged to a serotype different from O1a, suggest that these emerging serotype O1b strains can represent an important pathogenic group and could explain the low efficacy of the commercial vaccines. Moreover, it is known that the massive long-term use of a single vaccine can induce a strong selective pressure, resulting in the emergence of different virulent serotypes (Bachrach et al. 2001). Further work is required to determine the *in vivo* biological significance of Chilean serotype O1b *Yersinia ruckeri* strains, including virulence studies. Such information would benefit the development of vaccines prepared with antigens from different isolates that genuinely differ in cross protectiveness.

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LITERATURE CITED

Arias CR, Olivares-Fuster O, Hayden K (2007) First report of *Yersinia ruckeri* biotype 2 in the USA. J Aquat Anim Health 19:35–40

Austin B, Austin D (eds) (2007) Bacterial fish pathogens. Diseases of farmed and wild fish, 4th edn. Praxis Publishing, Chichester

Austin DA, Robertson PAW, Austin B (2003) Recovery of a new biogroup of *Yersinia ruckeri* from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum). Syst Appl Microbiol 26:127–131

Bachrach G, Zlotkin A, Hurvitz A, Evans DL, Eldar A (2001) Recovery of *Streptococcus iniae* from diseased fish previously vaccinated with a *Streptococcus* vaccine. Appl Environ Microbiol 67:3756–3758

Bravo S, Midtlyng PJ (2007) The use of fish vaccines in the Chilean salmon industry 1999–2003. Aquaculture 270: 36–42

Cipriano RC, Pyle JB, Starliper CE, Pyle SW (1985) Detection of *Vibrio anguilarum* antigen by dot blot assay. J Wildl Dis 21:211–218

CLSI (Clinical and Laboratory Standards Institute) (2009) Performance standards for antimicrobial disk susceptibility test, 10th edn. Approved Standard M02-A10. CLSI, Wayne, PA

Davies RL (1990) O-serotyping of *Yersinia ruckeri* with special emphasis on European isolates. Vet Microbiol 22: 299–307

Davies RL (1991) Clonal analysis of *Yersinia ruckeri* based on biotypes, serotypes and outer membrane protein-types. J Fish Dis 14:221–228

Davies RL, Frerichs GN (1989) Morphological and biochemical differences among isolates of *Yersinia ruckeri* obtained from wide geographical areas. J Fish Dis 12: 357–365

Dice LR (1945) Measures of the amount of ecological association between species. Ecology 26:297–302

Fouz B, Zarza C, Amaro C (2006) First description of non-motile *Yersinia ruckeri* serovar I strains causing disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum), cultured in Spain. J Fish Dis 29:339–346

Horne MT, Barnes AC (1999) Enteric redmouth disease (*Y. ruckeri*). In: Woo PTK, Bruno DW (eds) Fish diseases and disorders, Vol 3: viral, bacterial and fungal infections. CABI Publishing, Wallingford, p 455–477

Laemmli UK (1970) Cleavage of structural protein during assembly of the head of bacteriophage T4. Nature 227: 680–685

Osorio CR, Collins MD, Toranzo AE, Romalde JL (1999) 16S rRNA gene sequence analysis of *Photobacterium damselae* and nested-PCR method for rapid detection of fish pasteurellosis. Appl Environ Microbiol 65: 2942–2946

Romalde JL, Toranzo AE (1991) Evaluation of the API-20E system for the routine diagnosis of the enteric redmouth disease. Bull Eur Assoc Fish Pathol 11:147–149

Romalde JL, Margariños B, Barja JL, Toranzo AE (1993) Antigenic and molecular characterization of *Yersinia ruckeri*. Proposal for a new intraspecies classification. Syst Appl Microbiol 16:411–419

Romalde JL, Planas E, Sotelo JM, Toranzo AE (2003) First description of *Yersinia ruckeri* serotype O2 in Spain. Bull Eur Assoc Fish Pathol 23:135–138

Rucker R (1966) Redmouth disease of rainbow trout (*Salmo gairdneri*). Bull Off Int Epizoot 65:825–830

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sousa JA, Magariños B, Eiras JC, Toranzo AE, Romalde JL (2001) Molecular characterization of Portuguese strains of *Yersinia ruckeri* isolated from fish culture systems. J Fish Dis 24:151–159

Stevenson RMW, Airdrie DW (1984) Serological variation among *Yersinia ruckeri* strains. J Fish Dis 7:247–254

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA 4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Toledo MS, Troncoso M, Portell DP, Figueroa G (1993) Outbreak due to *Yersinia ruckeri* in cultured salmonids. Ann Microbiol 1:59–62

Toranzo A, Santos Y, Lemos ML, Ledo A, Bolinches J (1987) Homology of *Vibrio anguillarum* causing epizootics in turbot, salmon and trout reared on the Atlantic coast of Spain. Aquaculture 67:41–52

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of protein from polyacryalmide gel to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354

Troncoso M, Toledo MS, Portell DP, Figueroa G (1994) Aislamiento de *Yersinia ruckeri* en salmónidos de cultivos. Av Cienc Vet 9:122–127

Tsai CM, Frasch CE (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal Biochem 119:115–119

Versalovic J, Koeuth T, Lupski JR (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19: 6823–6831

Wheeler RW, Davies RL, Dalsgaard I, Garcia J and others (2009) *Yersinia ruckeri* biotype 2 isolates from mainland Europe and the UK likely represent different clonal groups. Dis Aquat Org 84:25–33

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Artículo:

Phenotypical and genetic characterization of *Yersinia ruckeri* strains isolated from recent outbreaks in farmed rainbow trout *Oncorhynchus mykiss* (Walbaum) in Peru.

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Resumen:

Un total de 30 cepas de *Yersinia ruckeri* causante de recientes brotes en sistemas de cultivo de truchas peruanos, se estudiaron mediante caracterizaciónes bioquímicas, serológicas, lipopolisacáridos (LPS), proteínas de membrana externa (OMP), y análisis de genotipado ERIC- y REP-PCR. Todos los aislados peruanos resultaron fermentativos, oxidasa positivos y negativos para la descarboxilación de la lisina, la ornitina y la utilización de glucosa y manitol, permitiendo su identificación presuntiva como *Y. ruckeri*. La secuenciación del gen 16S rRNA confirmó que los aislados eran realmente *Y. ruckeri* (> 99,98% de similitud). Aunque la mayoría de las cepas estudiadas fueron móviles y positivo para

producción de lipasa, correspondiendo al biotipo 1 de *Y. ruckeri*, 5 de estas cepas fueron negativas para ambas pruebas, siendo identificadas como del biotipo 2. Además, las pruebas de susceptibilidad a fármacos determinaron una alta sensibilidad a sulfametoxazol/trimetoprima, oxitetraciclina, ampicilina y enrofloxacina en todos los aislados. Serológicamente, todas las cepas peruanas estudiadas se identificaron como pertenecientes al serotipo O1 subgrupo a. El análisis de los perfiles de lipopolisacáridos (LPS), así como de proteínas de membrana totales y externa (OMP), y el correspondiente análisis de inmunoblot, apoyaron estos resultados. El genotipado realizado mediante ERIC y REP-PCR determinaron una mayor correlación de los aislados peruanos con la cepa NCIMB 2194^{T} independientemente del biotipo.

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Short communication

Phenotypical and genetic characterization of *Yersinia ruckeri* strains isolated from recent outbreaks in farmed rainbow trout *Oncorhynchus mykiss* (Walbaum) in Peru

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ABSTRACT

A total of 30 strains of *Yersinia ruckeri* causing recent outbreaks in Peruvian trout culture systems, were studied by means of biochemical characteristics, serology, lipopolysaccharide (LPS) and outer membrane protein (OMP) analysis, and ERIC and REP PCR fingerprinting. All the Peruvian isolates were found to be fermentative, oxidase negative and positive for decarboxylation of lysine and ornithine and utilization of glucose and mannitol, allowing their presumptive identification as *Y. ruckeri*. Sequencing of the 16S rRNA gene confirmed that isolates were indeed *Y. ruckeri* (>99.98% identity). Although most of the strains studied were motile and lipase positive corresponding to the biotype 1 of *Y. ruckeri*, 5 of these strains were negative from both tests, being identified as biotype 2. In addition, drug susceptibility tests determined high sensitivity to sulfamethoxazole/trimethoprim, oxytetracycline, ampicillin and enrofloxacin in all the isolates. Serologically, all the Peruvian strains studied were identified as belonging to the serotype O1 subgroup a. Analysis of the lipopolysaccharide (LPS) as well as total and outer membrane proteins (OMPs) profiles and the correspondent inmunoblotting, supported these results. Genotyping performed by means of ERIC- and REP-PCR determined major correlation of the Peruvian isolates with the type strain NCIMB 2194^T regardless of the biotype.

1. Introduction

Yersinia ruckeri the etiological agent of enteric redmouth disease (ERM) is a serious disease causing significant economic losses in the salmonid farming industry. The pathogen *Y. ruckeri* was initially isolated from diseased trout in the Hagerman Valley, Idaho (USA) in the 1950s (Rucker, 1966). Over the last 25 years, the disease also spread rapidly in European countries, Australia, South Africa and South America (Austin and Austin; 2007; Tobback et al. 2007).

The species includes two biotypes where strains positive for motility and lipase activity are grouped into biotype 1, while biotype 2 strains are negative for both tests (Davies and Frerichs, 1989; Evenhuis et al., 2009). In addition, there is a great serological variability within *Y. ruckeri*, with several intraspecies classifications being proposed, comprising four (Romalde et al., 1993) or five (Davies, 1990) different O-serotypes. Biotype 1 strains of serotype O1a (Hagerman strain) and O2b (O'Leary strain) cause most epizootic outbreaks, being the serotype O1a predominant in cultured salmonids (Austin and Austin, 2007; O'Leary et al., 1979; Stevenson and Airdrie, 1984). However, it has been recently proposed that this statement is not the case, describing the existence of new clonal groups also with high virulence (Tinsley et al., 2011).

Although generally well controlled by means of vaccination and antibiotic treatment, outbreaks of this disease have been periodically observed, especially in endemic areas. Formulation of most ERM commercial vaccines is based only on serotype O1a (Hagerman strain) however different degrees of cross-protection among serotypes have been reported (Stevenson and Airdrie, 1984). However, the number of ERM reports in previously vaccinated salmonids in Europe and USA has increased, and some of these outbreaks were attributed to emergent non-motile *Y. ruckeri* isolates (Arias et al., 2007; Fouz et al., 2006).

Rainbow trout is an exotic species in Peru and was introduced from the USA in 1925. The development of rainbow trout culture in Peru during the last three decades, involving a large number of egg and fish importations, mainly from USA, was not accompanied by an evaluation of the sanitary conditions of Peruvian aquaculture facilities. To our knowledge, the only study on this topic was performed by Bravo and Kojagura (2004) who reported the *Y. ruckeri* detection by serological procedures during two surveys of the fish health status of hatcheries, carried out in 1998 and 2000 in Peru. Even though the disease has been diagnosed more than 25 years ago and commercial vaccines are

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available in major salmonid culture countries, vaccination is not used as preventive strategies in Peruvian farms.

In this study, an exhaustive phenotypical, molecular and geneical characterization of 30 *Y. ruckeri* strains isolated from outbreaks occurred during 2008 in different Peruvian farms was performed, allowing to evaluate the genetic variability among strains and to hypothesize whether they represent native strains or are the result of fish imports.

2. Materials and methods

2.1. Bacterial isolates

Peruvian strains were isolated from outbreaks occurred during 2008 in four different fish farms located in the central Andes of Peru at 3283 a 3900 m above sea level. Farms consist of concrete tanks and the freshwater is supplied from three different rivers, with water temperatures ranging from 8.5 to 13 °C and average value of pH = 7. Diseased rainbow trout (n = 154), with variable size (from 0.3 to 45.5 g) showed exophthalmia, dark pigmentation, hemorrhages in the mouth, eyes and around the vent as main external signals as well as petechiae in liver, visceral fat and pyloric ceca in internal organs.

For bacterial isolation, samples were aseptically collected from liver, spleen and kidney and directly streaked onto trypticase soy-agar (TSA; Difco Laboratories, Detroit, USA) and incubated at 25 °C for 24–48 h. Pure cultures were kept frozen at − 80 °C in tryptic soy broth (TSB; Difco) supplemented with 15% glycerol. Type strain of the species and representative strains of the different O-serotypes following the scheme of Romalde et al. (1993) were also included in all the studies for comparative purposes (Table 1).

2.2. Biochemical and serological characterization

Bacterial isolates were subjected to morphological, physiological and biochemical tests using classical tube and plate procedures and the taxonomic position of the isolates were determined mainly following the schemes of Austin and Austin (2007). In parallel, all isolates were identified by means of the API 20E, and API ZYM (BioMerieux, France) using saline solution (NaCl 0.85%) for the bacterial suspensions. The study of acid production from carbon sources was also tested with the API 50CH (BioMerieux, France). In addition, susceptibility of the isolates strains to sulfamethoxazole/trimethoprim (25 μg per disc), tetracycline (30), oxytetracycline (30), ampicillin (10) and enrofloxacin (5) was determined by the disc diffusion method.

For the confirmatory serological identification, the isolates were examined for ability to agglutinate cross-absorbed antiserum raised against the reference strains of different serotypes (O1a, O1b, O2a, O2b, O2c, O3 and O4) of *Y. ruckeri*, using both slide agglutination assays and dot blot analysis (Bastardo et al., 2011).

Table 1
Yersinia ruckeri strains used in this study.

Reference number strain		Source	Collection[a]
Type strain	NCIMB 2194^T	*Oncorhynchus mykiss* (USA)	NCIMB
O-serotype strains[b]			
O1a	11.4	*O. mykiss* (Norway)	D.P. Anderson
O1b	1533	*Salmo salar* (Norway)	T. Håstein
O2a	RS6	*Salvelinus fontinalis* (USA)	R.M.W. Stevenson
O2b	11.29	*O. tshawytcha* (USA)	D.P. Anderson
O2c	RS2	*O. mykiss* (USA)	R.M.W. Stevenson
O3	11.47	*O. mykiss* (USA)	T. Cook
O4	11.73	*O. mykiss* (USA)	T. Cook
Fresh isolates from			
Farm A	17, 26	*O. mykiss* (Peru)	Laboratory collection
Farm B	23, 28	*O. mykiss* (Peru)	Laboratory collection
Farm C	1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 19, 20, 24, 29, 30, 32.	*O. mykiss* (Peru)	Laboratory collection
Farm D	15, 27, A18, A20, A24, A25, 41II	*O. mykiss* (Peru)	Laboratory collection

[a] NCIMB, National Collection of Marine Bacteria; D.P. Anderson, National Fish Health Research Laboratory, Kearneysville, West Virginia; T. Hänstein, National Veterinary Institute, Oslo; R.M.W. Stevenson, University of Guelph; T. Cook, Dpt. Microbiology, University of Maryland.
[b] Romalde et al. (1993).

2.3. Lipopolysaccharide (LPS) and outer membrane protein (OMP) analysis

LPS and OMPs of the Peruvian isolates were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) combined with immunoblotting as previously described by Romalde et al. (1993). For immunological analysis, absorbed antisera against the reference strains of serotypes O1a, O1b and O2b were employed.

2.4. 16S rRNA gene sequencing

Total bacterial DNA was extracted from pure bacterial cultures using the Insta-Gene matrix (Bio-Rad, Madrid, Spain). The concentration of DNA was quantified spectrophotometrically and adjusted to a concentration of 100 ng/μl. 16S rRNA gene from DNA templates was amplified and sequenced following the procedures of Bastardo et al. (2011).

2.5. REP and ERIC-PCR genotyping

Repetitive extragenic palindromic (REP) and the enterobacterial repetitive intergenic consensus (ERIC) sequences from all *Y. ruckeri* isolates were amplified by PCR as previously described (Versalovic et al., 1991). The PCR products were electrophoresed in agarose gels (1.5% w/v), stained with ethidium bromide (2 μg/ml), and analyzed with the Diversity Database software (Bio-Rad) as described by Bastardo et al. (2011).

3. Results and discussion

The 30 Peruvian isolates evaluated were Gram-negative fermentative rods, oxidase negative and positive for lysine and ornithine decarboxylase, hydrolysis of gelatin and utilization of glucose and mannitol, which allow their presumptive identification as *Y. ruckeri*. The results from all standard biochemical tests are shown in Table 2. The main differential traits among the Peruvian isolates were motility, Voges–Proskauer reaction, lipase, fermentation of sorbitol and utilization of citrate. Such variation in these biochemical tests has

Table 2
Differential biochemical characteristics observed for the Peruvian strains.

Test	Motility	Tween 80	Voges–Proskauer	Gelatin	Citrate	Sorbitol
Peruvian isolates	V (25)	V (11)	V (7)	+	V (16)	V (17)
Reference strain						
NCMB 2194^T	+	+	−	+	−	−
O-serotype reference strains						
11.4(O1a)	+	+	−	−	−	−
1533(O1b)	+	+	−	−	+	−
RS2(O2a)	+	+	+	−	+	+
11.29(O2b)	+	+	−	+	+	+
RS6(O2c)	+	+	+	+	−	+
11.47(O3)	+	+	+	+	+	+
11.73(O4)	+	+	−	−	−	−

^{T}: Type strain; +: positive reaction; −: negative reaction; V: variable reaction; (n): number of positive strains.

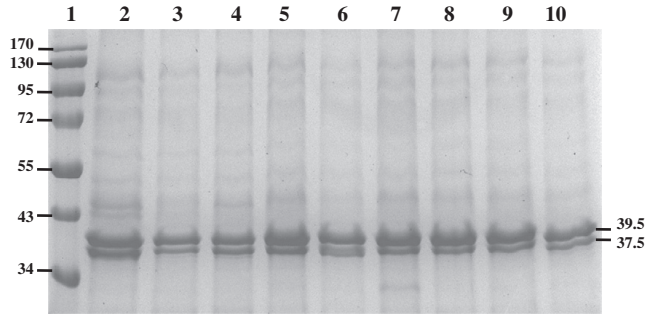

Fig. 1. OMP profiles of the *Y. ruckeri* strains studied. Lanes: 1, molecular size marker; 2, NCIMB 2194^T; 3–8, Peruvian isolates (the 30 isolates analyzed showed the same profile); 9, 11.4 (O1a); 10, 1533 (O1b). Numbers on the left indicate molecular size of markers (kDa). Numbers on the right indicate molecular size of major protein bands (kDa).

been reported in other studies for *Y. ruckeri* strains (Arias et al., 2007; Bastardo et al., 2011; Davies and Frerichs, 1989). Although most of the strains studied were motile and lipase positive corresponding to the biotype 1 established for *Y. ruckeri*, 5 of these strains were identified belonging to biotype 2 being non motile and lipase negative, which is interesting because *Y. ruckeri* biotype 2 had not been previously reported from Peru. The fact that both biotype 1 and 2 strains have been isolated from the recent Peruvian ERM outbreaks, indicate that in vivo studies are required to determine the real pathogenic relevance of these non-motile *Y. ruckeri* strains.

In the API 20E system 22 Peruvian isolates showed the same numeric profile (5104100) that *Y. ruckeri* type strain NCIMB^T 2194 and the other 8 isolates exhibited a different profile (5105100), which has also been reported for *Y. ruckeri* in previous studies (Topic et al., 2007). False negative reactions in API 20E reactions were found for VP, citrate and sorbitol. The results obtained using the miniaturized systems API ZYM and API 50CH were totally homogeneous among all the isolates analyzed, including the type strain, and matched with previous descriptions (Bastardo et al., 2011), which suggest that these systems could be useful as taxonomic tools in the diagnosis of *Y. ruckeri*. In addition, all isolates showed a drug-susceptibility similar to the *Y. ruckeri* type strain being highly sensitive to sulfamethoxazole/trimethoprim, oxytetracycline, ampicillin and enrofloxacin.

Results obtained by serological assays revealed that whole cell and thermostable O-antigens of all Peruvian isolates showed strong agglutination and immune reaction only with antiserum against the serotype O1 subgroup a (11.4 strain), supporting the findings of Bravo and Kojagura (2004). In addition, all the Peruvian isolates

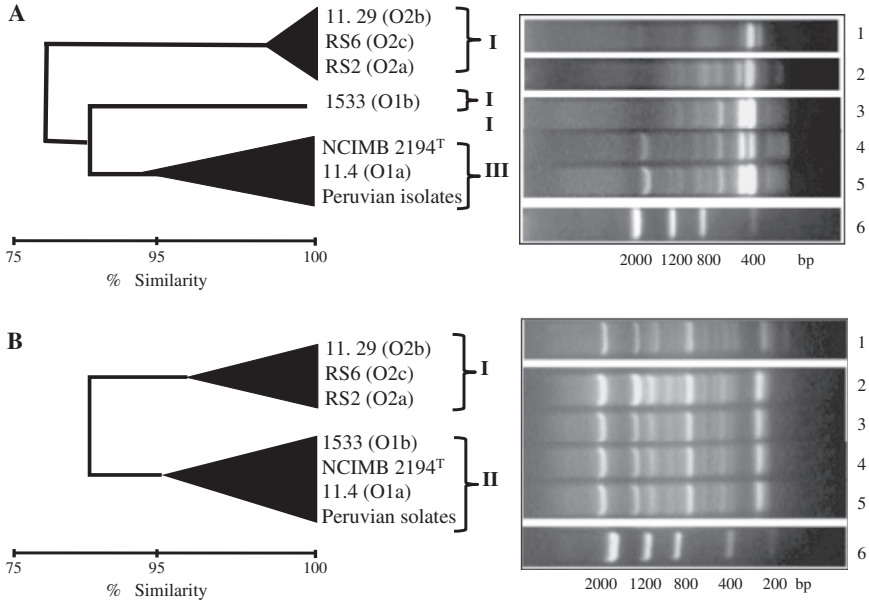

Fig. 2. Cluster obtained using Dice similarity coefficient and UPGMA analysis based on REP-PCR (A) and ERIC-PCR (B) produced patterns in *Y. ruckeri* strains. Numbers on the left indicate: 1, 11.29, RS6 and RS2 profile; 2, 1533; 3, NCIMB 2194^T; 4, 11.4; 5, Peruvian isolates profile (the thirty isolates analyzed showed the same pattern); 6, base pair size (bp) marker. Numbers below indicate bp size of markers.

showed the same LPS banding profile and was practically identical to the serotype O1a representative strain (11.4) and the *Y. ruckeri* type strain NCIMB 2194^T patterns. The immunoblot assays of the LPS confirmed the presence of a single serological group in these isolates, showing reactivity only when the antiserum raised against the 11.4 strain (serotype O1a) was used in the western blot (data not shown). Similar results were obtained for OMP, regardless of the biotype, showing a pattern (Fig. 1) consistent with OMP-type 3 established for *Y. ruckeri* (Davies, 1991).

Sequencing of the 16S rRNA gene confirmed the identification of the thirty Peruvian isolated, being determined the highest similarity (>99.96 %) with *Y. ruckeri* type strain (EF179132). Genotyping by ERIC-PCR showed discernible patterns for all *Y. ruckeri* isolates, consistent of 8–10 bands ranging from 250 to 1700 bp. Cluster analysis revealed that all Peruvian isolates and the reference serotype O1a strain (11.4) shared an identical ERIC-PCR profile, clustering with *Y. ruckeri* type strain (NCIMB 2194^T) at 91% similarity. In addition, serotype O1b (1533) and O2 (11.29 and RS6) strains included in ERIC-PCR analysis, grouped with Peruvian isolates cluster at 85% and 78% similarity, respectively (Fig. 2A). The band profiles obtained by REP-PCR were more homogeneous, consisting in 9–11 amplification bands ranging from 150 to 2600 bp. Two clusters were detected (Fig. 2B). Cluster I included the all the Peruvian isolates, the *Y. ruckeri* type strain, as well as strain of serotypes O1a and O1b, whereas serotype O2 strains were compiled in the cluster II. The similarity between the two clusters was 88%. Although, other authors reported genetic variability between isolates of *Y. ruckeri* biotype 1 and biotype 2 by PFGE (Arias et al., 2007; Ström-Bestor et al., 2010; Wheeler et al., 2009), genetic variability among the *Y. ruckeri* strains evaluated in this study was related only with the serotype and not the biotype.

Since *Y. ruckeri* was previously reported in Peru only after two fish health surveys conducted in 1998 and 2000 respectively, it is quite difficult to determine when, or from what source, this pathogen may have been introduced, although serological characterization, study of cell envelope components (LPS and OMP) and genotyping performed in this study, included all the Peruvian *Y. ruckeri* strains in the same clonal group 5 established for the Hagerman strain (Davies, 1991). These findings indicate, on the other hand, that the intensity of the *Y. ruckeri* infections in Peruvian farms could be greatly reduced through vaccination procedures using the ERM commercial vaccine.

Acknowledgments

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References

Arias, C.R., Olivares-Fuster, O., Hayden, K., 2007. First report of *Yersinia ruckeri* biotype 2 in the USA. J. Aquat. Anim. Health 19, 35–40.
Austin, B., Austin, D.A., 2007. Bacterial Fish Pathogens: Disease of Farmed and Wild Fish, 4th ed. Springer-Praxis.
Bastardo, A., Bohle, H., Ravelo, C., Toranzo, A.E., Romalde, J.L., 2011. Serological and molecular heterogeneity among *Yersinia ruckeri* strains isolated from farmed Atlantic salmon *Salmo salar* in Chile. Dis. Aquat. Org. 93, 207–214.
Bravo, S., Kojagura, V., 2004. First isolation of *Yersinia ruckeri* from rainbow trout (*Oncorhynchus mykiss*) in Peru. Bull. Eur. Ass. Fish Pathol. 24, 104–108.
Davies, R.L., 1990. O-serotyping of *Yersinia ruckeri* with special emphasis on European isolates. Vet. Microbiol. 22, 299–307.
Davies, R.L., 1991. Clonal analysis of *Yersinia ruckeri* based on biotypes, serotypes and outer membrane protein-types. J. Fish Dis. 14, 221–228.
Davies, R.L., Frerichs, G.N., 1989. Morphological and biochemical differences among isolates of *Yersinia ruckeri* obtained from wide geographical areas. J. Fish Dis. 12, 357–365.
Evenhuis, J.P., LaPatra, S.E., Verner-Jeffreys, D.W., Dalsgaard, I., Welch, T.J., 2009. Identification of flagellar motility genes in *Yersinia ruckeri* by transposon mutagenesis. Appl. Environ. Microbiol. 75, 6630–6633.
Fouz, B., Zarza, C., Amaro, C., 2006. First description of non-motile *Yersinia ruckeri* serovar I strains causing disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum), cultured in Spain. J. Fish Dis. 29, 339–346.
Laemmli, U.K., 1970. Cleavage of structural protein during assembly of the head of bacteriophage T4. Nature 227, 680–685.
O'Leary, P.J., Rohovec, J.S., Fryer, J.L., 1979. A further characterization of *Yersinia ruckeri* (enteric redmouth bacterium). Fish Pathol. 14, 71–78.
Romalde, J.L., Margariños, B., Barja, J.L., Toranzo, A.E., 1993. Antigenic and molecular characterization of *Yersinia ruckeri*. Proposal for a new intraspecies classification. Syst. Appl. Microbiol. 16, 411–419.
Rucker, R., 1966. Redmouth disease of rainbow trout (*Salmo gairdneri*). Bull. Off. Int. Epiz. 65, 825–830.
Stevenson, R.M.W., Airdrie, D.W., 1984. Serological variation among *Yersinia ruckeri* strains. J. Fish Dis. 7, 247–254.
Ström-Bestor, M., Mustamäki, N., Heinikainen, S., Hirvelä-Koski, V., Verner-Jeffreys, D., Wiklund, T., 2010. Introduction of *Yersinia ruckeri* biotype 2 into Finnish fish farms. Aquaculture 308, 1–5.
Tinsley, J.W., Austin, D.A., Lyndon, A.R., Austin, B., 2011. Novel non-motile phenotypes of *Yersinia ruckeri* suggest expansion of the current clonal complex theory. J. Fish Dis. 34, 311–317.
Tooback, E., Decostere, A., Hermans, K., Haesebrouck, F., Chiers, K., 2007. *Yersinia ruckeri* infections in salmonid fish. J. Fish Dis. 30, 257–268.
Topic Popovic, N., Coz-Rakovac, R., Strunjak-Perovic, I., 2007. Commercial phenotypic tests (API 20E) in diagnosis of fish bacteria: a review. Vet. Med.-Czech 52, 49–53.
Versalovic, J., Koeuth, T., Lupski, J.R., 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucl. Acids Res. 19, 6823–6831.
Wheeler, R.W., Davies, R.L., Dalsgaard, I., Garcia, J., Welch, T., Wagley, S., Bateman, K., Verner-Jeffreys, D., 2009. *Yersinia ruckeri* biotype 2 isolates from mainland Europe and the UK likely represent different clonal groups. Dis. Aquat. Org. 84, 25–33.

Artículo:

A polyphasic approach to study the intraspecific diversity of *Yersinia ruckeri* strains isolated from recent outbreaks in salmonid culture.

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Resumen:

Se llevó a cabo un análisis polifásico en cepas de *Yersinia ruckeri* aisladas recientemente de brotes en peces vacunados usando una combinación de diferentes métodos de caracterización fenotípica y molecular, con el fin de estudiar su variabilidad y las relaciones epidemiológicas. Se estudiaron ochenta cepas mediante técnicas de biotipado usando pruebas convencionales y el sistema API 20E, serotipado, perfiles de proteína de membrana externa (OMP) y lipopolisacáridos (LPS), y genotipado mediante las técnicas de ERIC-PCR y REP-PCR. Las cepas mostraron una alta diversidad, como se evidenció por la formación de diferentes grupos fenotípicos relacionados principalmente con los serotipos y los perfiles de LPS y OMP. La diversidad entre todos los aislados, calculada por el índice de diversidad de Simpson (*Di*), varió entre 0,35 (REP-

PCR) y 0,70 (OMP). Los valores más discriminativos (valor para *Di* > 0,86) se obtuvieron a partir de cualquier combinación de tres métodos que incluyan biotipo, serotipo, codigo de API 20E y, pérfil LPS o OMP. Con la combinación de todos los métodos de caracterización utilizados se obtuvo un valor *Di* de 0,90. Se evidenció asociación entre distintos grupos y las especies de huésped. Además, se observó que cepas con características similares estuvieron asociadas con brotes recientes ocurridos en peces vacunados en determinadas zonas geográficas. Nuestros resultados enfatizan la utilidad del uso de una combinación de varios métodos de tipado diferentes en los estudios epidemiológicos de diversidad y bacteriana.

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A polyphasic approach to study the intraspecific diversity of *Yersinia ruckeri* strains isolated from recent outbreaks in salmonid culture

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ABSTRACT

A polyphasic analysis was carried out on *Yersinia ruckeri* strains isolated from recently outbreaks in vaccinated fish using a combination of different phenotypic and molecular typing methods in order to study their variability and epidemiological relationships. Eighty strains were subjected to biotyping with conventional tests and API 20E system, serotyping, outer membrane protein (OMP) and lipopolysaccharide (LPS) profiling, and genetic fingerprinting by ERIC-PCR and REP-PCR techniques. The strains showed a high diversity, as evidenced by the formation of different phenotypic groups mainly related to the serotypes, LPS and OMP profiles. The diversity among all isolates, calculated as Simpson's diversity index (*Di*), varied between 0.35 (REP-PCR) and 0.70 (OMP). The most discriminative values (*Di* value $\geq$0.86) were obtained from any combination of three methods including biotype, serotype, API 20E profile, LPS or OMP. With the combination of all typing methods used a *Di* value of 0.90 was obtained. Association between different groups to the host species was evidenced. Furthermore, it seems that strains with similar characteristics are associated with recent outbreaks occurred in vaccinated fish in certain geographical areas. Our results emphasize the usefulness of using a combination of several different typing methods for epidemiological and bacterial diversity studies.

1. Introduction

Yersinia ruckeri is the causative agent of red mouth disease (ERM) or Yersinosis, which mainly affects salmonid fish, and causes heavy losses to their culture. *Y. ruckeri* strains comprise several serotypes with different subgroups (Stevenson and Airdrie, 1984; Romalde et al., 1993). The species also includes two biotypes, being strains positive for motility and lipase activity grouped into biotype 1, while biotype 2 strains are negative for both tests (Davies and Frerichs, 1989). However, *Y. ruckeri* infections have been mostly associated with the serotype O1a motile isolates. A wide variety of typing methods has been used for the characterization of *Y. ruckeri* strains in an attempt to find reliable markers within the species. Joint analysis of biotype and outer membrane protein (OMP) profile allowed the establishment of clonal groups (Davies, 1991). Furthermore, molecular techniques such as plasmid profiles, random amplification of polymorphic DNA (RAPD), pulse field gel electrophoresis (PFGE) and multilocus enzyme electrophoresis (MLEE) have been used to study the genetic variability of the species providing better insights in the relation between similar *Y. ruckeri* strains responsible for ERM outbreaks in the salmonid aquaculture (De Grandis and Stevenson, 1982; Schill et al., 1984; Garcia et al., 1998; Lucangeli et al., 2000; Wheeler et al., 2009). ERM has been successfully controlled for decades by vaccination with monovalent killed whole cell commercial vaccines. However, recent ERM outbreaks have occurred in vaccinated fish at farms in the southern United States and throughout Europe associated to an emerging non-motile group within the

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serotype O1a (Austin et al., 2003; Fouz et al., 2006; Wheeler et al., 2009; Ström-Bestor et al., 2010), for which commercial vaccines proved to be of limited efficacy (Deshmukh et al., 2012).

In the present study, we have studied 80 strains of *Y. ruckeri* using a combination of seven different typing methods involving phenotyping, serotyping and genotyping, in order to investigate the diversity and epidemiological relations of *Y. ruckeri* isolates from outbreaks of ERM occurred recently in trout farmed in USA, South America and Europe.

2. Materials and methods

2.1. Bacterial strains

The *Y. ruckeri* strains used in this study (see Fig. 1) included 71 isolates from diseased fish in different ERM outbreaks occurred between 2005 and 2009 in Chile, USA, and Portugal (vaccinated fish), as well as Peru and Finland (non-vaccinated fish). The *Y. ruckeri* reference strains NCIMB2194^T and NCIMB1316, as well as representative strains for all recognized serotypes (Romalde et al., 1993) were also included. All the isolates have been previously confirmed as belonging to *Y. ruckeri* by specific PCR or 16S rRNA sequencing (Arias et al., 2007; Ström-Bestor et al., 2010; Tobback et al., 2010; Bastardo et al., 2011a,b). Part of the biochemical, serological and molecular characterization of the Chilean, Peruvian and North-American isolates has been previously reported (Arias et al., 2007; Bastardo et al., 2011a,b). Isolates were routinely cultured and purified on tryptic soy agar (TSA; Difco Laboratories, Detroit, MI, USA) and were kept frozen at −80 °C in tryptic soy broth (TSB; Difco) supplemented with 15% glycerol. Bacteria were grown in trypticase soy agar (TSA; Difco) with incubation at 25 °C for 48 h.

2.2. Phenotypic and serological characterization

All isolates were subjected to morphological, physiological and biochemical test and the taxonomic identification of the isolates were determined mainly following the schemes of Austin and Austin (2007). The strains were assigned to biotype 1 or 2, according to the motile/lipase positive capacity or non-motile/lipase negative activity respectively. In addition, cultures were identified by means of the API 20E (BioMerieux, France) miniaturized system using saline solution (NaCl 0.85%) for the bacterial suspensions. Strips were incubated at 25 °C, and readings were performed from 24 to 48 h.

For the serological identification, slide agglutination and dot blot assays were performed with whole cells and O-antigens (Bastardo et al., 2011a; Cipriano et al., 1985) using antisera raised against the reference strains of the different serotypes (O1a, O1b, O2a, O2b, O2c, O3 and O4) of *Y. ruckeri* obtained as previously described (Romalde et al., 1993). For these analyses, the different antisera used were previously absorbed with the heterologous strains as described Stevenson and Airdrie (1984).

2.3. Molecular typing

Lipopolysaccharide and outer membrane protein (OMP) analysis were characterized in all *Y. ruckeri* strains by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) combined with immunoblotting as described by Romalde et al. (1993). For immunological analysis, absorbed antisera against the reference strains of serotypes O1a, O1b and O2b were employed.

2.4. Genotypic characterization

Genomic DNA was extracted from pure bacterial cultures using Insta-Gene matrix (Bio-Rad, Madrid, Spain). The concentration of DNA was quantified spectrophotometrically and adjusted to a concentration of 100 ng/μl.

For the genotyping, repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) sequences from all *Y. ruckeri* isolates were amplified by PCR and visualized as previously described (Bastardo et al., 2011a; Versalovic et al., 1991). Patterns were analyzed using the Diversity Database software (Bio-Rad). Similarities among isolates were estimated by means of the Dice coefficient (S_d) (Dice, 1945), and the dendrograms were generated using the unweighted pair-group (UPGMA) method.

2.5. Calculation of diversity indexes

In order to obtain the diversity index for each typing method and their different combinations, diversity values (*Di*) based on Simpson's diversity index, were calculated as described by Hunter and Gaston (1988). A high value for *Di* means that most isolates belong to different types, and a low value indicates that one type is dominant.

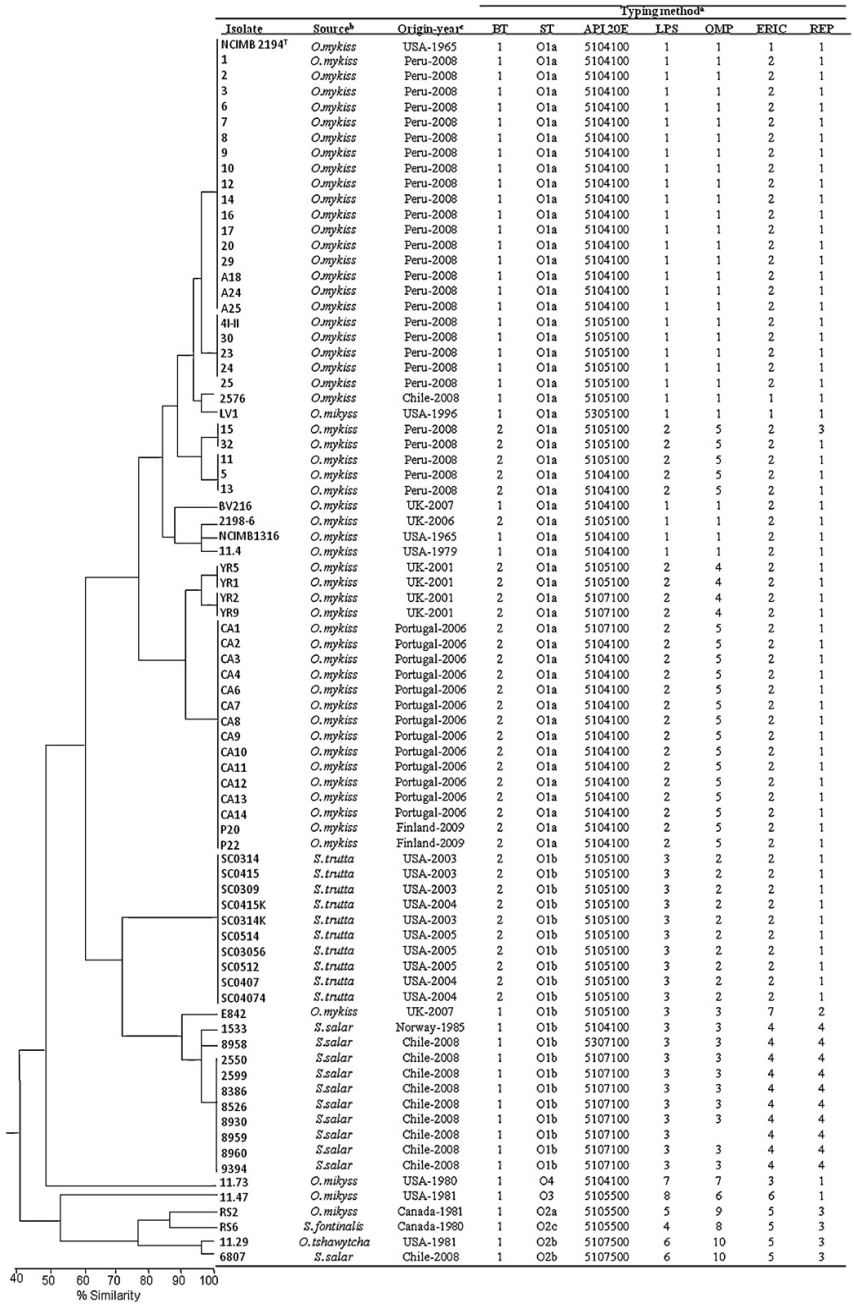
2.6. Polyphasic analysis

Composite data sets from the seven typing method performed were used to create a polyphasic profile for all isolates of *Y. ruckeri*. Phenotypic (biotype, serotype and API 20E) molecular (LPS and OMP profiles) and genotypic (ERIC-REP PCR patterns) data were introduced into a computerized database and analyzed using the software package Bionumerics v 4.0. Clustering of similarity coefficients was performed according to the UPGMA method.

3. Results

3.1. Phenotypic and molecular characterization

Table 1 shows the variation in the biochemical characteristics observed among the *Y. ruckeri* isolates analyzed in this study. The main differential traits among the isolates were motility, Voges–Proskauer reaction, lipase, fermentation of sorbitol, hydrolysis of gelatin and utilization of citrate. Although most of the strains studied (56.25%) were motile corresponding to the biotype 1 established for *Y. ruckeri*, 43.75% of the isolates were



Isolate	Source[b]	Origin-year[c]	Typing method[a] BT	ST	API 20E	LPS	OMP	ERIC	REP
NCIMB 2194[T]	*O.mykiss*	USA-1965	1	O1a	5104100	1	1	1	1
1	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
2	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
3	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
6	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
7	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
8	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
9	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
10	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
12	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
14	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
16	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
17	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
20	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
29	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
A18	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
A24	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
A25	*O.mykiss*	Peru-2008	1	O1a	5104100	1	1	2	1
4I-II	*O.mykiss*	Peru-2008	1	O1a	5105100	1	1	2	1
30	*O.mykiss*	Peru-2008	1	O1a	5105100	1	1	2	1
23	*O.mykiss*	Peru-2008	1	O1a	5105100	1	1	2	1
24	*O.mykiss*	Peru-2008	1	O1a	5105100	1	1	2	1
25	*O.mykiss*	Peru-2008	1	O1a	5105100	1	1	2	1
2576	*O.mykiss*	Chile-2008	1	O1a	5105100	1	1	1	1
LV1	*O.milkyss*	USA-1996	1	O1a	5305100	1	1	1	1
15	*O.mykiss*	Peru-2008	2	O1a	5105100	2	5	2	3
32	*O.mykiss*	Peru-2008	2	O1a	5105100	2	5	2	1
11	*O.mykiss*	Peru-2008	2	O1a	5105100	2	5	2	1
5	*O.mykiss*	Peru-2008	2	O1a	5104100	2	5	2	1
13	*O.mykiss*	Peru-2008	2	O1a	5104100	2	5	2	1
BV216	*O.mykiss*	UK-2007	1	O1a	5104100	1	1	2	1
2198-6	*O.mykiss*	UK-2006	2	O1a	5105100	1	1	2	1
NCIMB1316	*O.mykiss*	USA-1965	1	O1a	5104100	1	1	2	1
11.4	*O.mykiss*	USA-1979	1	O1a	5104100	1	1	2	1
YR5	*O.mykiss*	UK-2001	2	O1a	5105100	2	4	2	1
YR1	*O.mykiss*	UK-2001	2	O1a	5105100	2	4	2	1
YR2	*O.mykiss*	UK-2001	2	O1a	5107100	2	4	2	1
YR9	*O.mykiss*	UK-2001	2	O1a	5107100	2	4	2	1
CA1	*O.mykiss*	Portugal-2006	2	O1a	5107100	2	5	2	1
CA2	*O.mykiss*	Portugal-2006	2	O1a	5104100	2	5	2	1
CA3	*O.mykiss*	Portugal-2006	2	O1a	5104100	2	5	2	1
CA4	*O.mykiss*	Portugal-2006	2	O1a	5104100	2	5	2	1
CA6	*O.mykiss*	Portugal-2006	2	O1a	5104100	2	5	2	1
CA7	*O.mykiss*	Portugal-2006	2	O1a	5104100	2	5	2	1
CA8	*O.mykiss*	Portugal-2006	2	O1a	5104100	2	5	2	1
CA9	*O.mykiss*	Portugal-2006	2	O1a	5104100	2	5	2	1
CA10	*O.mykiss*	Portugal-2006	2	O1a	5104100	2	5	2	1
CA11	*O.mykiss*	Portugal-2006	2	O1a	5104100	2	5	2	1
CA12	*O.mykiss*	Portugal-2006	2	O1a	5104100	2	5	2	1
CA13	*O.mykiss*	Portugal-2006	2	O1a	5104100	2	5	2	1
CA14	*O.mykiss*	Portugal-2006	2	O1a	5104100	2	5	2	1
P20	*O.mykiss*	Finland-2009	2	O1a	5104100	2	5	2	1
P22	*O.mykiss*	Finland-2009	2	O1a	5104100	2	5	2	1
SC0314	*S.trutta*	USA-2003	2	O1b	5105100	3	2	2	1
SC0415	*S.trutta*	USA-2003	2	O1b	5105100	3	2	2	1
SC0309	*S.trutta*	USA-2003	2	O1b	5105100	3	2	2	1
SC0415K	*S.trutta*	USA-2004	2	O1b	5105100	3	2	2	1
SC0314K	*S.trutta*	USA-2003	2	O1b	5105100	3	2	2	1
SC0514	*S.trutta*	USA-2005	2	O1b	5105100	3	2	2	1
SC03056	*S.trutta*	USA-2005	2	O1b	5105100	3	2	2	1
SC0512	*S.trutta*	USA-2005	2	O1b	5105100	3	2	2	1
SC0407	*S.trutta*	USA-2004	2	O1b	5105100	3	2	2	1
SC04074	*S.trutta*	USA-2004	2	O1b	5105100	3	2	2	1
E842	*O.mykiss*	UK-2007	1	O1b	5105100	3	3	7	2
1533	*S.salar*	Norway-1985	1	O1b	5104100	3	3	4	4
8958	*S.salar*	Chile-2008	1	O1b	5307100	3	3	4	4
2550	*S.salar*	Chile-2008	1	O1b	5107100	3	3	4	4
2599	*S.salar*	Chile-2008	1	O1b	5107100	3	3	4	4
8386	*S.salar*	Chile-2008	1	O1b	5107100	3	3	4	4
8526	*S.salar*	Chile-2008	1	O1b	5107100	3	3	4	4
8930	*S.salar*	Chile-2008	1	O1b	5107100	3	3	4	4
8959	*S.salar*	Chile-2008	1	O1b	5107100	3		4	4
8960	*S.salar*	Chile-2008	1	O1b	5107100	3	3	4	4
9394	*S.salar*	Chile-2008	1	O1b	5107100	3	3	4	4
11.73	*O.milkyss*	USA-1980	1	O4	5104100	7	7	3	1
11.47	*O.milkyss*	USA-1981	1	O3	5105500	8	6	6	1
RS2	*O.milkyss*	Canada-1981	1	O2a	5105500	5	9	5	3
RS6	*S.fontinalis*	Canada-1980	1	O2c	5105500	4	8	5	3
11.29	*O.tshawytcha*	USA-1981	1	O2b	5107500	6	10	5	3
6807	*S.salar*	Chile-2008	1	O2b	5107500	6	10	5	3

Fig. 1. UPGMA clustering of all *Y. ruckeri* isolates studied using results from combined typing with 7 different methods. [a], biotype (BT), serotype (ST), according to Romalde et al. (1993), lipopolysaccharide (LPS), outer membrane protein (OMP), ERIC-PCR and REP-PCR. [b], host fish species: *Oncorhynchus mykiss*, *Salmo trutta*, *Salmo salar*, *Salvelinus fontinalis*, *Oncorhynchus tshawytcha*. [c], Country-year of isolation.

Table 1
Differential biochemical and serological characteristics observed among *Y. ruckeri* strains.

	N	O-serotypes	Motility	VP	Gelatin	Citrate	Sorbitol	Lipase
Origin of isolates								
Chile	11	O1a, O1b, O2b	+	+	+	V (2)	V (1)	+
Peru	27	O1a	V (25)	V (7)	+	V (19)	V (17)	V (11)
Portugal	13	O1a	–	V (10)	–	–	–	–
UK	7	O1a, O1b	V (2)	V (2)	V (1)	V (3)	–	V (1)
USA	11	O1a, O1b	V (1)	–	V (1)	–	–	–
Finland	2	O1a	–	V (1)	–	–	–	–
Reference strains								
NCIMB2194[T]		O1a	+	–	+	–	–	+
NCIMB1316		O1a	+	–	+	–	–	+
O-serotype representative strains[a]								
11.4		O1a	+	–	–	–	–	+
1533		O1b	+	–	–	+	–	+
RS2		O2a	+	+	–	+	+	+
11.29		O2b	+	–	+	+	+	+
RS6		O2c	+	+	+	–	+	+
11.47		O3	+	+	+	+	+	+
11.73		O4	+	–	–	–	–	+

[T], Type strain; +, positive reaction; –, negative reaction; V, variable reaction; (*n*), number of positive strains.
[a] Romalde et al. (1993).

identified as belonging to biotype 2. These non-motile strains were isolated from Peru (5 isolates), Finland (2 isolates), Portugal (13 isolates), UK (5 isolates), and USA (10 isolates). Seven different profiles were recorded among the isolates using the API 20E system. The most common profiles were 5104100 and 5105100, which corresponded approximately to 54.2% and 25.3% of the strains, respectively.

Agglutination assays performed using whole cells and thermostable O-antigens revealed the presence of 3 different major serotypes among the *Y. ruckeri* strains examined in this study (Fig. 1). All the isolates gave strong agglutination with homologous antisera. The majority of isolates were identified as belonging serotypes O1a and O1b (67.5 and 26.5% respectively). The results of the dot blot test support those of slide agglutination (data not shown). Although most of the *Y. ruckeri* strains ascribed to biotype 2 belonged to serotype O1a (25 isolates), 10 non-motile strains isolates from USA were identified as serotype O1b.

Eight different profiles were observed among the LPS of all *Y. ruckeri* strains. The LPS profiles obtained for all the isolates studied were similar to those shown by the representative strains of serotypes O1a, O1b and O2b (Fig. 2A). Interestingly, within serotype O1a the LPS patterns exhibited by the biotype 1 and biotype 2 isolates showed slight differences. It was observed in biotype 1 isolates that the inter-band spacing was higher than biotype 2 strains with more bands in the 34–55 kDa region. Conversely, no differences in the LPS profile between biotype 1 and biotype 2 strains identified as belonging to serotype O1b were observed.

The SDS-PAGE analysis of the OMPs revealed that the isolates yielded 10 different patterns (Fig. 2B). Although the majority of the OMPs profiles could be related to the serotype of the isolates, differences between banding profiles of OMPs among biotype 2 isolates were detected. The OMP profile for serotype O1a/biotype 2 *Y. ruckeri* strains isolated from Portugal, Peru and UK showed different OMP patterns in comparison to the reference serotype O1a strain (11.4) in which the expression of a 36 kDa relatively abundant porin protein was weakly present in the biotype 2 strains. Similar findings were observed between the serotype O1b/biotype 2 strains isolated from USA and the motile reference serotype O1b strain in which the same band corresponding to the 36 kDa porin protein was also very weakly detected. The western blot assay of total and OMPs showed correspondence with the dot blot and LPS results (data not shown).

3.2. Genotypic characterization

All the *Y. ruckeri* strains were typeable, rendering discernible amplification patterns by any of the PCR methods used.

Genotyping by REP-PCR allowed the identification of profiles with 9–11 amplification bands ranging from 150 to 2200 bp that originated four genetics groups (Fig. 2C). Cluster 1 included the majority of isolates and grouped biotypes 1 and 2 *Y. ruckeri* strains from serotype O1a, non-motile USA O1b isolates, as well as serotypes O3 and O4 strains. Cluster 2 included the UK motile O1b strain. Serotype O2 subgroups a, b and c were grouped in cluster 3, while cluster 4 was formed by the motile serotype O1b isolates.

The analysis of ERIC-PCR fingerprints showed profiles with bands (between 8 and 10) with molecular weights from 50 to 1500 bp (Fig. 2D). Seven different genetic groups were identified on the basis of the band profiles. Cluster 1 grouped only serotype O1a/biotype 1 isolates including the *Y. ruckeri* type strain NCIMB2174[T], while cluster 2 contained both motile and non-motile serotype O1a (from Portugal, Finland and UK) and O1b (from USA and Chile) isolates. These clusters showed 85% similarity. The remaining groups were formed by the serotype O4 strain, serotype O1b reference strain together with motile

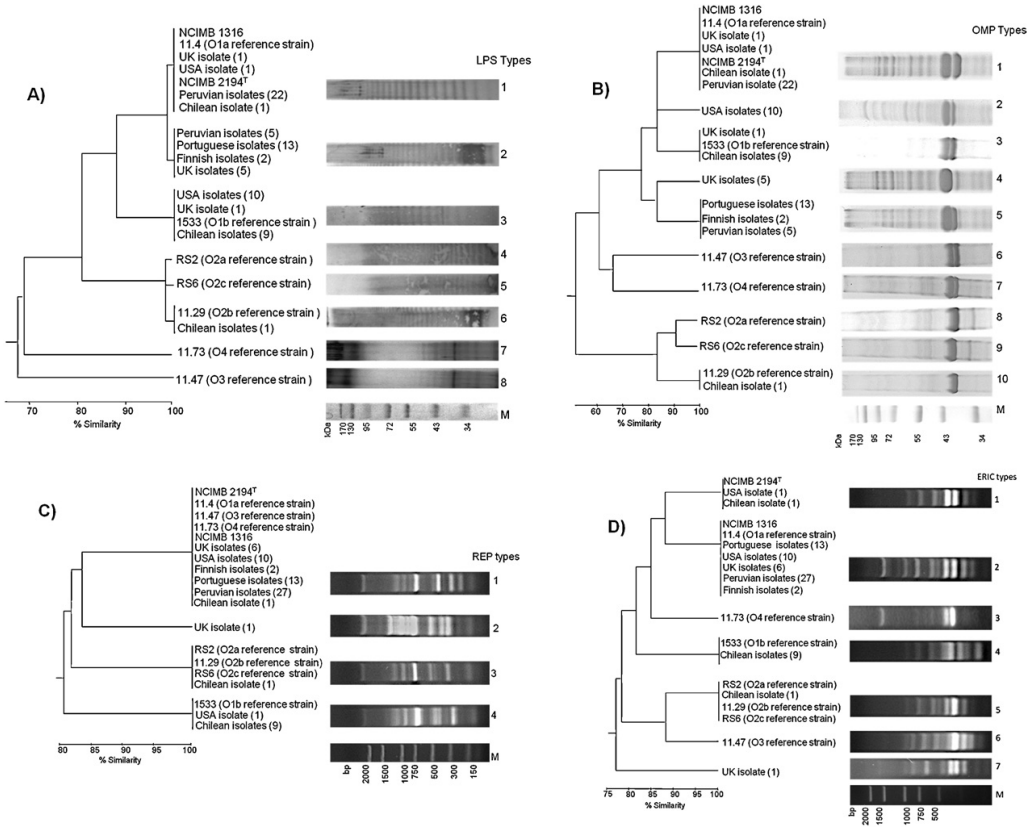


Fig. 2. Dendrograms obtained using Dice similarity coefficient and UPGMA analysis based on lipopolysaccharide (A), outer membrane protein (B), ERIC-PCR (C) and REP-PCR (D) produced patterns for *Y. ruckeri* strains. M, base pair size or kDa molecular weight marker.

serotype O1b Chilean isolates, serotype O2 (with subgroups a, b and c) isolates, serotype O3 strain and, a unique profile exhibited by one O1b motile isolate from UK, respectively.

3.3. Diversity among Y. ruckeri isolates

The *Di* was calculated for all strains and seven different typing methods. Diversity values varied between 0.35 for REP-PCR and 0.76 for OMPs typing (Table 2). The other five methods yielded diversities between 0.35 and 0.74. The *Di* obtained with the combination of all the typing methods calculated for all isolates was 0.90. Considering the heterogeneous profiles described for *Y. ruckeri* using API 20E system, the diversity without including these profiles were also calculated resulting in a *Di* value of 0.83. In general, the most discriminative value was obtained from any combination of three of the following methods BT, ST, API 20E, LPS or OMP typing (*Di* values between 0.86 and 0.88) Furthermore, ERIC-PCR and REP-PCR typing could each rendering an acceptable *Di* value (between 0.73 and 0.76) when are combined with ST and LPS or ST and OMP typing methods.

3.4. Polyphasic analysis

In order to show the diversity of *Y. ruckeri* strains in this study all the results obtained using the different typing methods were combined in a single similarity matrix that was then used to generate a dendrogram (Fig. 1). The upper part of the dendrogram shows a high diversity among the serotype O1a isolates including several subgroups that appear to be related to the biotype and/or geographical origin. The middle part of the dendrogram shows the formation of another cluster with all the strains of serotype O1b, also differentiated into two subgroups according to biotype of these strains. The lower part of this figure shows heterogeneous groups formed by two isolates that belong to serotype O2b and the remaining serotypes O2 (subgroups a and c), O3 and O4 represented each by one isolate.

Table 2
Discriminatory indices (*Di*) of the typing methods and different combinations used in this study.

Typing methods	Number of types	*Di*
Biotype (BT)	2	0.49
Serotype (ST)	7	0.50
API 20E (API)	9	0.74
Lipopolysaccharide profile (LPS)	8	0.71
Outer membrane protein profile (OMP)	9	0.76
ERIC-PCR fingerprint (ERIC)	6	0.39
REP-PCR fingerprint (REP)	4	0.35
BT + ST	9	0.74
BT + ST + API	14	0.86
BT + ST + LPS	14	0.86
BT + ST + OMP	18	0.86
BT + API + LPS	16	0.86
BT + API + OMP	18	0.87
BT + LPS + OMP	18	0.86
ST + API + LPS	16	0.86
ST + API + OMP	18	0.87
ST + LPS + OMP	18	0.86
ST + LPS + REP	10	0.73
ST + LPS + ERIC	12	0.74
ST + OMP + REP	18	0.76
ST + OMP + ERIC	19	0.76
BT + ST + API + LPS	16	0.87
BT + ST + API + LPS + OMP	18	0.88
BT + ST + API + LPS + OMP + ERIC	22	0.89
BT + ST + API + LPS + OMP + ERIC + REP	23	0.90
BT + ST + LPS + OMP + ERIC + REP	14	0.83

4. Discussion

Classically, typing of *Y. ruckeri* strains for taxonomic or epidemiological purposes has been performed by means of phenotypic analysis, including biochemical and serological test, as well as by molecular and genetic techniques including LPS and OMP analysis, ribotyping, PFGE and MLEE (De Grandis and Stevenson, 1982; Davies, 1991; Romalde et al., 1993; Garcia et al., 1998; Wheeler et al., 2009) Although the variability observed in the biochemical and serological characteristics has allowed to establish different schemes for the intraspecific classification of *Y. ruckeri*, several studies have independently reported a high degree of homogeneity among *Y. ruckeri* serotype O1a strains (Schill et al., 1984; Furones et al., 1993). In the present study, a high differentiation at intraspecific level has been determined among *Y. ruckeri* strains recently isolated from outbreaks in different geographical areas using a polyphasic approach.

Three typing methods (API 20E, LPS and OMP profiles) yielded "medium-high" *Di* values ranging between 0.71 and 0.74. However, the highest discriminatory values within the species ($Di > 0.86$) were obtained when the combinations of these 3 or more typing methods were used. These results suggest that API 20E, LPS and OMP profiles were valuable typing method for *Y. ruckeri* strains specially in combination with other techniques applied in this study. Similar results have been reported for *Y. ruckeri* isolated from Turkey where antibiotyping, SDS-PAGE and RAPD-PCR profiles were analyzed (Onuk et al., 2011).

On the other hand, Tinsley et al. (2011) reported eight different phenotypes among 63 *Y. ruckeri* isolates based on combinations of the biochemical, serological and OMP types, grouping 3 phenotypes the serovar O1/biotype 2 isolates. Contrarily, in this study the variability observed among the serotype O1a (equivalent to serovar O1) strains was higher resulting in 5 and 8 different profiles for biotypes 1 and 2 respectively. These differences can be due to the variability provided by genotyping methods included in the present study. However, different groups were also established among biotypes 1 and 2, O1b serotype strains, which means the first evidence of variability within this serotype.

In previous studies, biotype 2 *Y. ruckeri* strains from UK and Europe were associated with the clonal group 2 (Davies, 1991; Wheeler et al., 2009), highlighting that the ERM outbreaks were only associated with serotype O1a clonal groups 2 and 5. The present study provides evidence of the existence of new clonal groups, as biotype 2 can be observed among other OMPs and serological types (in addition to O1a serotype), supporting the need of expansion of the clonal group theory established by Davies (1991).

Phenotypic variability within the *Y. ruckeri* strains isolated from recent outbreaks in vaccinated fish was observed in this study regardless to the serotype. However, certain association to the geographical origin was observed. Thus, *Y. ruckeri* strains causing ERM disease isolated from Chile, USA, Portugal and UK formed specific groups. The isolates causing outbreaks in non-vaccinated fish from Peru were included in three different groups. These results support those from other recent publications on *Y. ruckeri* that have also demonstrated the dominance of some subgroups in certain areas (Wheeler et al., 2009; Tinsley et al., 2011; Welch et al., 2011).

The association between different groups to the host species such as Atlantic salmon, brown trout and rainbow trout was evidenced in the polyphasic analysis performed in this study. Further investigations are required to evaluate the *in vivo* biological significance of the physiological, serological and genetic variability observed in *Y. ruckeri* isolates from the different host species. Particularly, experimental confirmation that isolates from recent outbreaks have increased in virulence is required. This information could benefit the development of new improved vaccines that confer effective cross-protection against the wide range of pathogenic *Y. ruckeri* isolates for which the commercials vaccines commonly used have failed (Deshmukh et al., 2012).

In conclusion, using the results from seven typing methods in combination, the diversity of strains within the species *Y. ruckeri* was very high. In addition, it has been shown that isolates with similar characteristics were related with certain fish species, and/or predominated in some geographical areas. It is important to point out that these observations could only be evidenced through the use of combined data from phenotypic, molecular and genetic typing, highlighting the usefulness of the joint analysis of several typing methods for bacterial diversity and epidemiological studies.

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References

Arias, C.R., Olivares-Fuster, O., Hayden, K., 2007. First report of *Yersinia ruckeri* biotype 2 in the USA. J. Aquat. Anim. Health 19, 35–40.

Austin, B., Austin, D.A., 2007. Bacterial fish pathogens. Diseases of farmed and wild fish, 4th ed. Praxis Publishing, Chichester.

Austin, D.A., Robertson, P.A.W., Austin, B., 2003. Recovery of a new biogroup of *Yersinia ruckeri* from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum). Syst. Appl. Microbiol. 26, 127–131.

Bastardo, A., Bohle, H., Ravelo, C., Toranzo, A.E., Romalde, J.L., 2011a. Serological and molecular heterogeneity among *Yersinia ruckeri* strains isolated from farmed Atlantic salmon *Salmo salar* in Chile. Dis. Aquat. Organ. 93, 207–214.

Bastardo, A., Sierralta, V., León, J., Ravelo, C., Romalde, J.L., 2011b. Phenotypical and genetic characterization of *Yersinia ruckeri* strains isolates from recent outbreaks in farmed rainbow trout *Onchorhyncus mykiss* (Walbaum) in Peru. Aquaculture 317, 229–232.

Cipriano, R.C., Pyle, J.B., Starliper, C.E., Pyle, S.W., 1985. Deteccion of *Vibrio anguilarum* antigen by dot blot assay. J. Wildl. Dis. 21, 211–218.

Davies, R.L., 1991. Clonal analysis of *Yersinia ruckeri* based on biotypes, serotypes and outer membrane protein types. J. Fish Dis. 14, 221–228.

Davies, R.L., Frerichs, G.N., 1989. Morphological and biochemical differences among isolates of *Yersinia ruckeri* obtained from wide geographical areas. J. Fish Dis. 12, 357–365.

De Grandis, S.A., Stevenson, R.M.W., 1982. Variation in plasmid profiles and growth characteristics of *Yersinia ruckeri* strains. FEMS Microbiol. Lett. 15, 199–202.

Deshmukh, S., Raida, M.K., Dalsgaard, I., Chettri, J.K., Kania, P.W., Buchman, K., 2012. Comparative protection of two different commercial vaccines against *Yersinia ruckeri* serotype O1 and biotype 2 in rainbow trout (*Oncorhynchus mykiss*). Vet. Immunol. Immunopathol. 145, 379–385.

Dice, L.R., 1945. Measures of the amount of ecological association between species. Ecology 26, 297–302.

Fouz, B., Zarza, C., Amaro, C., 2006. First description of non-motile *Yersinia ruckeri* serovar I strains causing disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum), cultured in Spain. J. Fish Dis. 29, 339–346.

Furones, M.D., Rodgers, C.J., Munn, C.B., 1993. *Yersinia ruckeri*, the causal agent of enteric redmouth disease (ERM) in fish. Ann. Rev. Fish Dis. 3, 105–125.

Garcia, J.A., Dominguez, L., Larsen, J.L., Pedersen, K., 1998. Ribotyping and plasmid profiling of *Yersinia ruckeri*. J. Appl. Microbiol. 85, 949–955.

Hunter, P.R., Gaston, M.A., 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. 26, 2465–2466.

Laemmli, U.K., 1970. Cleavage of structural protein during assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lucangeli, C., Morabito, S., Capriol, A., Achenea, L., Busania, L., Mazzolinib, E., Fabrisb, E., Macrì, A., 2000. Molecular fingerprinting of strains of *Yersinia ruckeri* serovar O1 and *Photobacterium damsela* subsp. *piscicida* isolated in Italy. Vet. Microbiol. 76, 273–281.

Onuk, E., Ciftci, A., Findik, G., Ciftci, G., Altun, S., Balta, F., Ozer, S., Coban, A., 2011. Phenotypic and molecular characterization of *Yersinia ruckeri* isolates from rainbow trout (*Onchorynchus mykiss*, Walbaum, 1972) in Turkey. Berl. Munch. Tierarztl. Wochenschr. 124, 320–328.

Romalde, J.L., Margariños, B., Barja, J.L., Toranzo, A.E., 1993. Antigenic and molecular characterization of *Yersinia ruckeri*. Proposal for a new intraspecies classification. Syst. Appl. Microbiol. 16, 411–419.

Schill, W.B., Phelps, S.R., Pyle, S.W., 1984. Multilocus electrophoretic assessment of the genetic structure and diversity of *Yersinia ruckeri*. Appl. Environ. Microbiol. 48, 975–979.

Stevenson, R.M.W., Airdrie, D.W., 1984. Serological variation among *Yersinia ruckeri* strains. J. Fish Dis. 7, 247–254.

Ström-Bestor, M., Mustamäki, N., Heinikainen, S., Hirvelä-Koski, V., Verner-Jeffreys, D., Wiklund, T., 2010. Introduction of *Yersinia ruckeri* biotype 2 into Finnish fish farms. Aquaculture 308, 1–5.

Tinsley, J.W., Austin, D.A., Lyndon, A.R., Austin, B., 2011. Novel non-motile phenotypes of *Yersinia ruckeri* suggest expansion of the current clonal complex theory. J. Fish Dis. 34, 311–317.

Tobback, E., Hermans, K., Decostere, A., Van den Broeck, W., Haesebrouck, F., Chiers, K., 2010. Interactions of virulent and avirulent *Yersinia ruckeri* strains with isolated gill arches and intestinal explants of rainbow trout *Oncorhynchus mykiss*. Dis. Aquat. Organ. 90, 175–179.

Versalovic, J., Koeuth, T., Lupski, J.R., 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 19, 6823–6831.

Welch, T.J., Verner-Jeffreys, D., Dalsgaard, I., Wiklund, T., Evenhuis, J.P., Garcia Cabrera, J.A., Hinshaw, J.M., Drennan, J.D., La Patra, S.E., 2011. Independent emergence of *Yersinia ruckeri* biotype 2 in the United States and Europe. Appl. Environ. Microbiol. 77, 3493–3499.

Wheeler, R.W., Davies, R.L., Dalsgaard, I., Garcia Cabrera, J., Welch, T.J., Wagley, S., Bateman, K.S., Verner-Jefrey, D.W., 2009. Characterization of geographically and temporally diverse *Yersinia ruckeri* isolates: evidence that UK and mainland European biotype 2 isolates represent different clonal groups. Dis. Aquat. Organ. 84, 25–33.

4. CAPÍTULO II:

ESTRUCTURA POBLACIONAL, HISTORIA DEMOGRÁFICA Y FILOGEOGRAFÍA DE *Yersinia ruckeri.*

Artículo:

Multilocus sequence typing reveals high genetic diversity and epidemic population structure for the fish pathogen *Yersinia ruckeri.*

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Resumen:

Yersinia ruckeri es el agente causante de la enfermedad entérica de la boca roja en peces y es uno de los principales patógenos bacterianos causantes de pérdidas en la acuicultura de salmónidos. Previos métodos de tipado, incluyendo el análisis de enzimas de restricción, electroforesis de campo pulsado y la electroforesis en gel de enzimas multilócicas (MLEE) han indicado una estructura de población clonal. En este trabajo, se describe un esquema de tipado de secuencias multilócicas (MLST) para *Y. ruckeri,* basado en la secuencia de fragmentos internos de seis genes escenciales. Este esquema MLST se aplicó a 103 cepas de *Y. ruckeri* de diversas áreas geográficas, hospedadores, así como de origen ambiental. Las secuencias obtenidas a partir de este trabajo se depositaron en una base de datos pública (http://publmst.org/yruckeri/) y están disponibles. Se identificaron treinta

tipos de secuencias diferentes (ST), 21 de las cuales estuvieron representadas por un solo aislado, lo que evidenció una alta diversidad genética. El ST2 agrupó a más de un tercio de los aislados y se observó con mayor frecuencia entre los aislados de truchas. Se identificaron dos grandes complejos clonales (CC) mediante el análisis de eBURST, mostrando un origen evolutivo común para los 94 aislados que formaron los 21 STs dentro del CC1, y otro para los 6 aislados de los 6 STs dentro del CC2. También se asoció a algunos ST únicos con los aislados de brotes recientes en peces salmónidos vacunados.

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Multilocus sequence typing reveals high genetic diversity and epidemic population structure for the fish pathogen *Yersinia ruckeri*

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Summary

***Yersinia ruckeri* is the causative agent of enteric redmouth in fish and one of the major bacterial pathogens causing losses in salmonid aquaculture. Previously typing methods, including restriction enzyme analysis, pulsed-field gel electrophoresis and multilocus enzyme electrophoresis (MLEE) have indicated a clonal population structure. In this work, we describe a multilocus sequence typing (MLST) scheme for *Y. ruckeri* based on the internal fragment sequence of six housekeeping genes. This MLST scheme was applied to 103 *Y. ruckeri* strains from diverse geographic areas and hosts as well as environmental sources. Sequences obtained from this work were deposited and are available in a public database (http://pubImst.org/yruckeri/). Thirty different sequence types (ST) were identified, 21 of which were represented by a single isolate, evidencing high genetic diversity. ST2 comprised more than one-third of the isolates and was most frequently observed among isolates from trout. Two major clonal complexes (CC) were identified by eBURST analysis showing a common evolutionary origin for 94 isolates forming 21 STs into CC1 and for 6 isolates of 6 STs in the CC2. It was also possible to associate some unique ST with isolates from recent outbreaks in vaccinated salmonid fish.**

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Introduction

The Gram-negative bacterium *Yersinia ruckeri* is recognized as the aetiological agent of enteric redmouth (ERM) disease, a systemic infection of salmonid fish that causes important economic losses worldwide. *Yersinia ruckeri*, which belongs to the family *Enterobacteriaceae* (Ross *et al.*, 1966) was initially isolated from rainbow trout, *Oncorhynchus mykiss* (Walbaum), in the Hagerman Valley of Idaho, USA, in the 1950s and is now widely found in all trout producing countries (Austin and Austin, 2007). Infection results in a septicaemic condition with haemorrhages in the mouth, on the body surface and internal organs.

Two biotypes have been described for this bacterial species. Biotype 1 strains are positive for motility and lipase activity, whereas biotype 2 strains are negative for both tests (Davies and Frerichs, 1989). In addition, the species has been grouped into 6 serovars (Stevenson and Airdrie, 1984) or 4 O-serotypes with different subgroups (Romalde *et al.*, 1993) by using different serotyping systems. Strains of serotype O1a (classic serovar I) caused most epizootic outbreaks in cultured salmonid (Austin and Austin, 2007).

Enteric redmouth has been used successfully controlled for decades by vaccination with commercial monovalent killed whole cell vaccines. Although formulations of most commercial vaccines are based only on serotype O1a (Hagerman strain), different degrees of cross-protection among serotypes have been reported. In recent years, ERM vaccine breakdowns have been described in Europe and USA, mostly attributed to *Y. ruckeri* biotype 2 strains (Austin *et al.*, 2003; Fouz *et al.*, 2006; Arias *et al.*, 2007). Other epizootics have been reported in vaccinated Atlantic salmon (*Salmo salar*) from Chile caused by serotype O1b/biotype 1 strains (Bastardo *et al.*, 2011a).

Molecular techniques such as random amplification of polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE) and multilocus enzyme electrophoresis (MLEE) have been used to study the genetic variability of this species showing a low genetic intraspecies diversity. Schill and colleagues (1984) using MLEE observed only four electropherotypes for 47 isolates of *Y. ruckeri*,

Table 1. Genetic characteristics and evolutionary variation among the six loci included in the MLST scheme for *Y. ruckeri* (all 103 isolates).

Gene	Size of fragment amplified/used in MLST (bp)	Mean G+C content	Number of alleles	Number of polymorphic sites	*Pi*	d_N/d_S	*H*
glnA	493/416	50.5	9	7	0.0064	0.3427	0.2528
gyrB	503/454	45.6	6	8	0.0075	0.2238	0.3107
Y-HSP60	635/509	47.5	4	4	0.0039	0.0000	0.0577
recA	512/472	49.8	4	11	0.0125	0.1339	0.5773
dnaJ	1134/632	53.7	7	16	0.0078	0.0000	0.4717
thrA	693/303	48.2	4	3	0.0055	0.0000	0.4841

Pi, average number of nucleotide differences per site; d_N/d_S, ratio of mean non-synonymous substitutions per non-synonymous site/mean synonymous substitution per synonymous site; *H*, Nei's index of diversity.

indicating that the genetic structure of *Y. ruckeri* is clonal, with one predominant clonal group. Lucangeli and colleagues (2000) have studied the ribotypes, PFGE patterns and interspersed repetitive sequences (IRS)-PCR of 30 *Y. ruckeri* O1a strains, reporting a high level of genetic homogeneity for all the isolates. Other studies reported also high degree of homogeneity among *Y. ruckeri* O1a strains based on serology, LPS, OMP, total DNA fingerprints and plasmids profiles (De Grandis and Stevenson, 1982; Davies, 1991; Romalde *et al*., 1993). Wheeler and colleagues (2009) reported a total of 44 PFGE pulsotypes from among 160 isolates, providing better insights in the relationship between similar *Y. ruckeri* clones responsible for recent ERM outbreak among salmonid. More recently, Verner-Jeffreys and colleagues (2011) reported the heterogeneous assembly of types in serotype O1 *Y. ruckeri* strains with respect to pathogenicity and host, and Tinsley and colleagues (2011) suggested the need of expansion of the clonal group theory in this species, highlighting the existence of new clonal groups. However, the use of these techniques as typing methods is limited because of inter-laboratory reproducibility issues.

Multilocus sequence typing (MLST) was successfully applied for many bacterial species and is currently regarded as a gold-standard for typing, able to even replace PFGE and MLEE (Maiden, 2006). By MLST, the genes encoding the enzymes involved in conserved metabolic and information processes are compared at the DNA-sequence level. Being sequence-based, MLST provides a definitive characterization of bacterial isolates that is consistent among laboratories (Jolley and Maiden, 2010). Herein, we establish an MLST scheme for *Y. ruckeri*. The diversity and evolutionary relationships among a geographically and temporally diverse collection of isolates were investigated using this scheme.

Results

Nucleotide diversity

DNA sequence analysis of the six housekeeping genes resulted in a concatenated sequence of 2786 base pairs for phylogenetic analysis. A descriptive analysis of nucleotide and allele diversity for each locus is presented in Table 1. Allele sizes for the genes included in the MLST scheme varied between 303 bp (*thr*A) and 632 bp (*dna*J). A total of 34 different alleles were identified, 16 of which were synapomorphic (i.e. shared and derived). The numerical index of diversity (*H*) for each allele ranged from 0.0577 to 0.5773, with the lowest degree of diversity observed for Y-HSP60 (0.0577). The most polymorphic loci were *gln*A and *dna*J with 9 and 7 different alleles respectively. However, *dna*J and *gyr*B retained more synapomorphic alleles with 4 each. Mean G+C content varied from 45.6% (*gyr*B) to 53.7% (*dna*J). The G+C content of *Y. ruckeri* has been reported around 47.5% (Ewing *et al*., 1978), thus the G+C content of *dna*J alleles was at least 6.2% higher than the other partial gene sequence. The nucleotide diversity per site (*Pi*) ranged from 0.0039 to 0.0121, with the lowest degree of diversity observed for Y-HSP60 (0.0039).

Synonymous substitutions (d_S) occurred more frequently than non-synonymous substitutions in every gene (d_N). The d_N/d_S ratios calculated for each of six loci and for the concatenated sequences were < 1 (in particular Y-HSP60, *dna*J and *thr*A gave a d_N/d_S value of zero), indicating that these loci were subject to stabilizing or purification selection. Non-synonymous changes were not found in Y-HSP60 and *dna*J, which indicated that non-synonymous sites are evolving slower than synonymous sites. With randomized data sets (1000 trials) used, linkage disequilibrium was estimated with the I_A parameter from allelic profiles, a statistic that was expected to be zero when the alleles were in the linkage equilibrium (free recombination). Herein, the I_A value for the whole strain collection was 0.5563 ($P = 0.000$) that differs significantly from zero. When analysing the 30 unique STs only a much smaller I_A value (0.0244) ($P = 0.000$) was obtained. Standardized I^S_A value was 0.113 for all isolates and 0.021 when only STs were used (Table 2). In general, these data showed a non-random distribution of alleles in the *Y. ruckeri* population although, recombination may also be occurring within different subpopulations.

Table 2. Multilocus linkage disequilibrium analysis of the 103 *Y. ruckeri* isolates studied.

Group (*n*)	V_E	V_O	I_A	*P*	$\bar{r}_A$
Total isolates (103)	1.010	1.5727	0.5563	0.000	0.1113
Total STs (30)	1.206	1.3344	0.0244	0.000	0.0212

V_E, Values for expected variance; V_O, observed variance; I_A, index of association with associated *P* value and standardized index association $\bar{r}_A$.

Population structure: clonal complexes

The molecular characterization of the 103 *Y. ruckeri* isolates by MLST identified 30 different STs indicating genotypic diversity (Table 3). Twenty-one STs were represented by a single isolate, while 9 STs included between 2 and 43 isolates. ST 2 and ST 1 were the most frequent STs grouping 43 and 16 isolates respectively. Other STs also represented by more than one strain were ST 7 (8 isolates), ST 3 (4 isolates), ST 16 (3 isolates), and STs 9, 14, 23 and 26 (2 isolates each).

The 30 STs generated in this data set were separated by eBURST into two clonal complexes (CC1 and CC2) and three singletons using the default stringent criteria (5/6 shared alleles) (Fig. 1). CC1 comprised 91 strains within 21 STs. ST 14 was identified by eBURST as ST ancestral type or founder (Fig. 1) for this clonal complex (bootstrap group value of 80%). This founder comprised only two isolates and derived in 9 SLVs (ST 3, ST 4, ST 5, ST 7, ST 15, ST 18, ST 23, ST 26 and ST 30) and 7 DLVs. ST 2 and the DLV ST 1 (equivalent to a SLV of ST 2), emerged as successful subgroup founders (Fig. 1), each with its own cluster of linked SLVs. Sub-founder ST2 (bootstrap subgroup value of 95%) was the most frequent in the population (43 strains) and showed 8 SLVs and 11 DLVs. Sub-founder ST1 (bootstrap subgroup value of 87%) derived in 7 SLVs and 7 DLVs. CC2 contained six STs (ST 17, ST 21, ST 22, ST 24, ST 25 and ST 27) represented each for a single strain. ST 21 was identified as ancestral type of CC2 with 3 SLVs (ST 22, ST 25 and ST 27) and 1 DLV. Furthermore, ST 8, ST 19 and ST 20 were identified as singletons.

When the stringent SLV criterion was relaxed (4/6 shared alleles) STs formed a single network component (Fig. 1). Then, CC1 was connected to CC2 through 14 different DLVs, and singletons ST 8 and ST 20 were connected only to CC1 by 4 and 1 different DLVs respectively.

Quantification of recombination and mutation

Global genetic distance between isolates cannot reflect the variation of genetic relatedness across loci that result from recombination. However, identical ATs occurred in very different genetic context as a result of genetic exchanges across the *Y. ruckeri* population. The SLVs allele variants found between the two clonal complexes and the different subgroups identified by eBURST were analysed for the determination of the events responsible for their evolution. A total of 25 SLVs were identified within the 2 clonal complexes. In 12 of the 25 allelic comparisons the descendant allele differed from the ancestral allele at a single site; 4 of these single-nucleotide differences were synonymous. Five (41.6%) of these 12 alleles were novel and 7 (58.4%) were shared (i.e. they were found elsewhere in the data set). Of the 13 descendant alleles that differed at multiple ($\geq$ 2) sites, 7 (53.85%) were novel and 6 (46.15%) were shared.

Twenty out of 25 SLVs arose from a recombination event, whereas only 5 arose by mutation. This resulted in a per-allele r/m parameter of 4:1. In the case of the per-site analysis r/m parameter ratio was 7.5:1. These two parameters suggest that the initial steps of *Y. ruckeri* clonal diversification at allele or individual nucleotide sites are fourfold and sevenfold more likely to occur by recombination than by point mutation. Furthermore, the majority of the strains showed a star phylogeny radiating from the same central point in the split decomposition analysis (Fig. S1). A split graph based on the concatenated sequences displayed an interconnecting network structure rather than a single bifurcation tree (Fig. S2), which also suggested recombination.

The relative low genetic diversity in this analysis obliterated the chance of detecting recombination when DnaSP5 was used. With exception of *gyr*B ($R_{min} = 1$) recombination events were not detected. When computed on the concatenation of six loci, the R_{min} value was found to be 3, indicative of additional recombination between the consecutive loci.

Clustering and phylogenetic analysis

Allelic profile-based phylogenetic analysis was performed by using the UPGMA (unweighted-pair group method using average linkages) algorithm to explore the relationships among the 103 strains of *Y. ruckeri* studied. The UPGMA tree showed two major clusters A and B (Fig. 2 and Fig. S3). The majority of isolates (94 isolates) were in cluster A, while 6 isolates were in cluster B and 3 were not clustered. The division was concordant with population divisions based on eBURST analysis. Cluster A was inclusive of subpopulations grouped into CC1 by eBURST, and cluster B contained only subpopulation of the CC2. The strains not clustered in UPGMA tree were consistent with the singletons assigned by eBURST.

Geographical distribution and host fish species association of STs

Association between the genotype of the strains and the host fish species and geographical area was detected for

Table 3. Sequence types (ST) of *Y. ruckeri* strains obtained by MLST and relation with serotype, biotype, host, origin and year of isolation.

ST	Allele type	Serotype	Biotype	Host[c]	Origin	Year	No.
ST1	11 1 1 1 1	O1a[a]	1	*O. mykiss*	USA	1965	1
		O1a	1	*O. mykiss*	Peru	2008	7
		O1a	2	*O. mykiss*	Peru	2008	1
		O1a	2	*O. mykiss*	UK	2007	3
		O1a	1	*O. mykiss*	Denmark	1987	1
		O1a	1	*O. mykiss*	USA	1980	1
		O1a	1	*S. salar*	Chile	2008	1
		O1b	1	*S. salar*	Chile	2008	1
ST2	11 1 1 1 2	O1a[b]	1	*O. mykiss*	USA	1968	1
		O1a	1	*O. mykiss*	Peru	2008	14
		O1a	1	*O. mykiss*	Spain	1989	3
		O1a	2	*O. mykiss*	UK	2007	1
		O1a	2	*O. mykiss*	Portugal	2006	14
		O1a	2	*O. mykiss*	Finland	2009	2
		O1b	2	*S. trutta*	USA	2003–2005	7
ST3	12 1 1 2 2	O1a	1	Sediment	Portugal	1994	1
		O1b[b]	1	*S. salar*	Norway	1985	1
		O3	1	*O. mykiss*	Portugal	1994	1
		O3	1	Sediment	Portugal	1994	1
ST4	21 1 1 2 2	O2b	1	*O. tshawytcha*	USA	1981	1
ST5	11 2 1 2 2	O2a[b]	1	*O. mykiss*	Canada	1980	1
ST6	31 1 1 1 2	O3[b]	1	*O. mykiss*	USA	1981	1
ST7	11 1 1 2 1	O1b	1	*S. salar*	Chile	2008	8
ST8	12 3 1 3 1	O2b	1	*S. salar*	Chile	2008	1
ST9	13 1 1 1 1	O1a	2	*O. mykiss*	Peru	2008	2
ST10	41 1 1 1 1	O1a	1	*O. mykiss*	Peru	2008	1
ST11	14 1 1 1 1	O1a	2	*O. mykiss*	Peru	2008	1
ST12	13 1 1 1 2	O1a	2	*O. mykiss*	Peru	2008	1
ST13	12 1 1 1 1	O1b	1	*O. mykiss*	UK	1995	1
ST14	11 1 1 2 2	O1a	1	*O. mykiss*	UK	2007	1
		O1a	1	*O. mykiss*	USA	1996	1
ST15	51 1 1 2 2	O2b	1	*O. tshawytscha*	Canada	1989	1
ST16	61 1 1 1 2	O1b	2	*S. trutta*	USA	2004–2005	3
ST17	71 1 1 4 4	O1a	1	*S. salar*	Finland	1985	1
ST18	15 1 1 2 1	O1a	1	*O. mykiss*	Portugal	1994	1
ST19	86 4 4 5 2	O1a	1	Water	Portugal	1994	1
ST20	91 1 1 6 1	O2b	1	*O. mykiss*	Germany	1985	1
ST21	72 1 1 4 2	O1b	1	*S. malma*	Canada	1982	1
ST22	72 1 1 4 4	O2b	1	*A. anguilla*	Denmark	1987	1
ST23	11 1 1 6 2	O1a	1	*S. trutta*	Denmark	1983	1
		O2b	1	*O. mykiss*	Spain	2002	1
ST24	11 1 1 4 4	O1a	1	*O. zibethica*	Canada	1982	1
ST25	72 1 1 1 2	O1a	1	*O. mykiss*	Denmark	1983	1
ST26	15 1 1 2 2	O3	1	Sediment	Portugal	1994	1
		O3	1	*O. mykiss*	Portugal	1994	1
ST27	72 1 1 7 2	O4	1	*O. mykiss*	USA	1982	1
ST28	11 1 1 1 3	O1a	1	*O. mykiss*	Peru	2008	1
ST29	11 1 2 1 2	O4[b]	1	*O. mykiss*	USA	1981	1
ST30	11 1 3 2 2	O2b	1	*O. mykiss*	Scotland	2005	1

a. *Y. ruckeri* type strain.
b. Representative O-serotype strains.
c. Host fish species: *Oncorhynchus mykiss*, *Salmo trutta*, *Salmo salar*, *Salvelinus fontinalis*, *Oncorhynchus tshawytcha*.
Allele type cites the loci ordered: *glnA*, *gyrB*, Y-HSP60, *recA*, *dnaJ* and *thrA*.
No., number of strains.

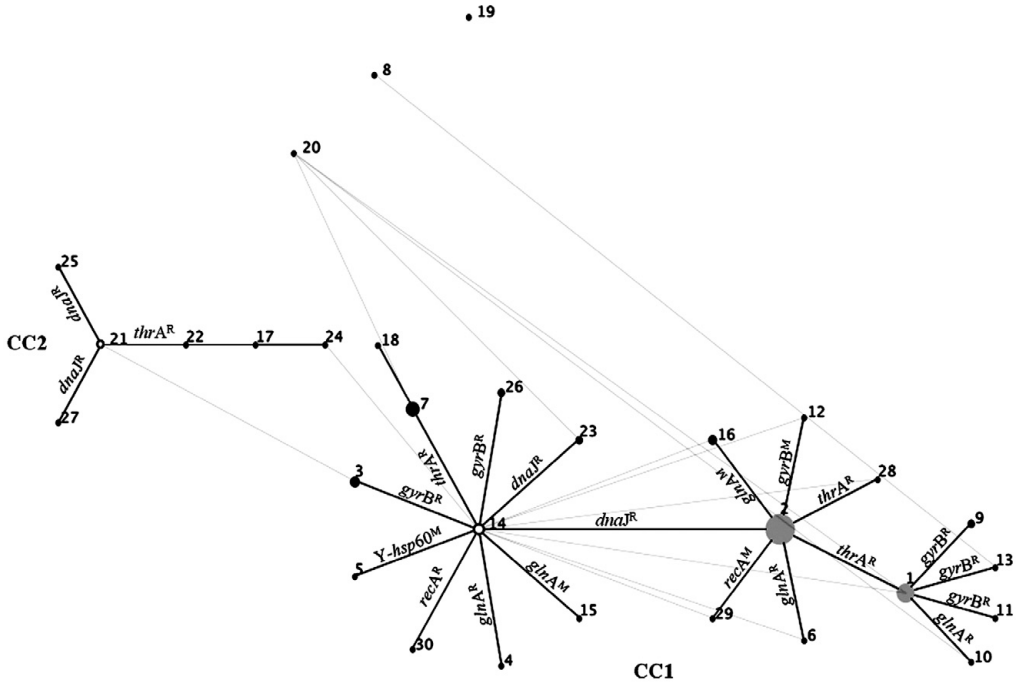

Fig. 1. Population snapshot of all the sequence types (ST), included in the MLST database for *Y. ruckeri*. The snapshot was made using the eBURST program. STs that are SLVs of each other are shown connected by black lines. DLV STs are shown connected by grey lines. Clonal complex (CC) is marked. The sizes of the circle are related to the number of strains within each ST. The founder and cofounder genotypes are represented by open circles and grey circles respectively. Recombination or mutation is indicated by a superscripted R or M respectively.

some STs. Particularly, the sub-founders ST 1 and ST 2 grouped most of strains isolated from salmonid species. Thereby, ST 2 grouped 43 strains (41.7%) isolated from *O. mykiss* in Peru, Europe (Spain, UK, Portugal and Finland) and USA during 1979–2009, and 7 strains (6.8%) isolated from *Salmo trutta* in USA (2003–2005). Interestingly, the majority of genetic variants descendant of this ST were also isolated from *O. mykiss* in Peru, USA and Spain, or from *S. trutta* in USA. Sub-founder ST 1 included besides the type strain of *Y. ruckeri* other 16 strains (15.5%) isolated from Atlantic salmon in Chile, or from rainbow trout in Peru, USA and Europe (UK, Denmark) from 1979 to 2009. The descendant STs of this sub-founder were also detected in Peru (ST 9, ST 10 and ST 11) or in UK (ST 13), but isolated exclusively from *O. mykiss*.

The STs grouped in CC2 represented by a single strain were associated to different host in different geographical areas. This clonal complex included founder ST 21 from *Salmo malma* in Canada, ST 17 from *S. salar* in Finland, ST 22 from *Anguilla anguilla* and ST 25 from *O. mykiss* in Denmark, ST 24 from *Ondatra zibethica* in Canada, and ST 27 from *O. mykiss* in USA. Singletons identified in the *Y. ruckeri* population were found dispersed in three very distant regions of the world, ST 8 from *S. salar* in Chile, ST 20 from *O. mykiss* in Germany and ST 19 from water in Portugal.

Relationship between serotypes, biotypes and STs

Serotyping and biotyping are customarily used for the characterization of *Y. ruckeri*. Representative strains of the six different serotypes and the two existing biotypes were included in the present study (Table 3). Biotype 1/serotype O1a were mostly found in *Y. ruckeri* strains. Biotype 2 was present only into strains of the serotype O1a and O1b. Frequently, strains belonging to genetically distant STs shared the same serotype and biotype. Serotype O1a was present in 12 and 3 STs of the CC1 and CC2 respectively. Conversely, isolates in the same ST displayed different serotypes. ST 3 compiled strains belonging to serotypes O1a, O1b and O3; ST1 and ST 2

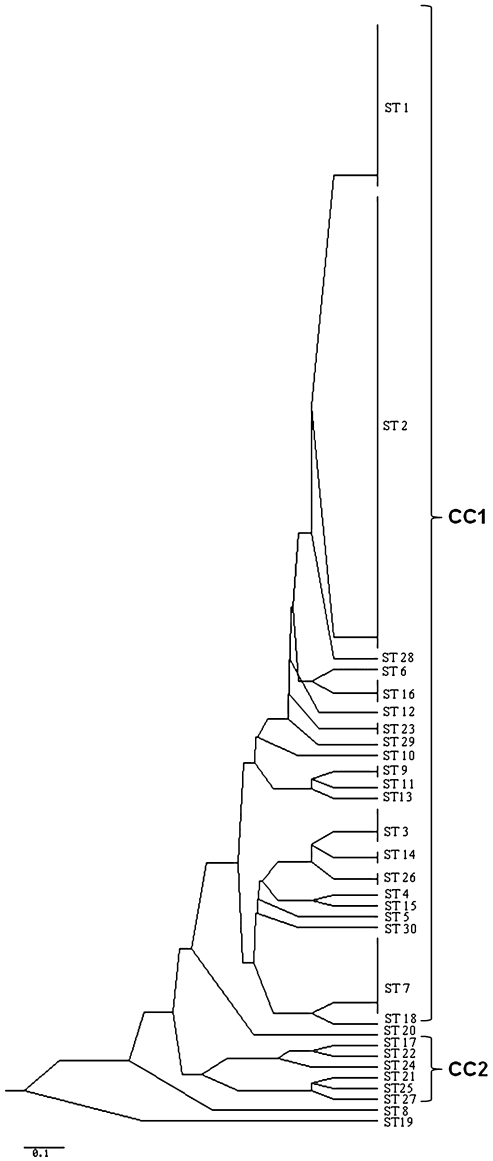

Fig. 2. UPGMA tree constructed from the profiles of the 103 strains of *Y. ruckeri*. The different sequence types (ST) were labelled in the tree. Scale represents the evolutionary distance. Another version of the tree with full strain labels is shown in Fig. S3.

included both O1a and O1b serotypes, and ST 23 consisted of O1b and O2b serotypes. Therefore, multiple serotypes were observed in each of the two clonal complexes.

On the other hand, sub-founder ST 2 grouped most strains belonging to biotype 2 (69.4%); the remaining biotype 2 strains were grouped into sub-founder ST 1 (30.6%). SLVs ST 12 and ST 16 originated from sub-founder ST 2, and SLVs ST 9, ST 11 and ST 13 formed from sub founder ST 1, comprised only biotype 2 strains. None of the strains of biotype 2 was found in the ancestral ST of the CC1 (ST 14), neither among strains grouped into the CC2 or in the singletons.

Discussion

This study describes an MLST scheme that was applied to a collection of *Y. ruckeri* strains, with diverse host and geographical origin, to investigate the evolutionary and genetic aspects of this bacterial pathogen. This MLST is based on the allelic profile variation of *gln*A, *gyr*B, *dna*J, Y-HSP60, *rec*A and *thr*A genes, which were demonstrated to be under purifying pressure ($d_N/d_S < 1$). This indicates a strong selection such that most amino acid substitutions are deleterious, as being typically observed for house-keeping genes (Perez-Losada *et al.*, 2006). Among the 103 *Y. ruckeri* strains used in this study; 30 different allelic combinations were established and nucleotide diversity per site (*Pi*) ranged from 0.0039 to 0.0121. Although these values indicate a relative low degree of genetic diversity for *Y. ruckeri*, they were higher than those reported for other fish pathogenic bacteria such as *Flavobacterium psycrophilum*, for which *Pi* values ranged from 0.0016 to 0.0094 (Nicolas *et al.*, 2008).

Recombination rates vary widely between different bacterial species, being detected high levels for marine and aquatic bacteria by means of the recombination/mutation ratio (Vos and Didelot, 2009). The results obtained indicated that recombinational replacement contributed 7.4 times more than point mutation to the genetic variations among *Y. ruckeri* isolates (ratio of 4:1 at the allele level, and 7.4:1 at the nucleotide level). These values are much higher than those reported for a mildly recombinogenic bacterium, such as *Escherichia coli* (per-allele ratio of 0.84:1 and per-nucleotide ratio of 5.18:1) (Salerno *et al.*, 2007) and somewhat lower than those of *F. psycrophilum* (per-allele ratio of 4.5:1 and per-nucleotide ratio of 26:1) (Nicolas *et al.*, 2008). Thus, recombination appears to play a greater role than mutation for the generation and maintenance of the genetic diversity within *Y. ruckeri*.

Almost half of the *Y. ruckeri* strains belonged to two STs (ST 1 and ST 2). These two STs belonged to the same clonal complex (CC1), which comprised most of the recognized STs (21 of 30). The persistence of these same STs over extended periods (e.g. ST 1 in numerous countries from 1965 to 2008 and ST 2 from 1979 to 2009) is indicative of a clonal population structure. However, a clonal population is composed of strains derived from a

common ancestor that diversified predominantly by mutation rather than recombination (Smith *et al*., 1993). The fourfold per-allele and sevenfold per-site frequencies of recombination events relative to mutations found in this study are uncharacteristic of a highly clonal population. These apparently contradictory results for *Y. ruckeri* clonality are consistent with those reported for *Vibrio parahaemolyticus* (Gonzalez-Escalona *et al*., 2008) and *Staphylococcus epidermidis* (Miragaia *et al*., 2007), for which an epidemic population structure has been proposed. Evidence of other clonal complex (CC2) and three STs singletons among the analysed strains constitutes the first definitive demonstration of a *Y. ruckeri* clonal complex other than the pandemic clonal complex (CC1) and further supports the epidemic model.

CC1 has been linked almost exclusively to outbreaks associated with culture of salmonid fish in the world since 1970s, while CC2 has been geographically and temporally restricted to USA, Denmark, and Canada (from 1980 to 1987). This finding indicates the homogeneous nature of the pandemic clonal complex (CC1), regardless the geographical site of isolation. Although, new variants are arising (sub-founders ST 2 and ST 1), they apparently have not yet become well established for replacing the ancestor type (ST 14). Thus, epidemic model is also consistent with the epidemiology of *Y. ruckeri*, which suggest that ERM started as a geographically isolate disease that relatively quickly became widely disseminated (Austin and Austin, 2007).

Wheeler and colleagues (2009) using PFGE reported a higher homology of the UK and European isolates of *Y. ruckeri* with USA isolates rather than each other, suggesting that this pathogen was introduced separately into the UK and mainland Europe from the USA, and that subsequent cross-border transfer of UK and European isolates has been limited. By MLST, the ST founder in the CC1 comprised two strains isolated separately from *O. mykiss* in UK and USA. Although ST 1 and ST 2, including most of the *Y. ruckeri* strains, coexist in USA, UK and Europe, the presence of other 10 STs specific for USA, other 10 STs unique in Europe and only one ST restricted to UK, strengthens the hypothesis that these isolates may have evolved independently in these areas. Such scenario could explain the high diversity of STs found in Europe and USA, including those observed in not salmonid fish species.

In this study, association between ST and host fish species were mainly observed in the sub-founders ST1 and ST2 and their derivate STs. Sub-founder ST 2 comprised exclusively *Y. ruckeri* strains isolated from *O. mykiss* and *S. trutta*. Similarly, sub-founder ST 1 was found associated exclusively to *O. mykiss* and *S. salar*. This finding might reflect an adaptive niche specialization supporting the hypotheses that the amplification and dissemination of *Y. ruckeri* occurred when this pathogen found an alternative niche (intensively cultured rainbow trout) more than by preferential routes of transmission (Welch *et al*, 2011).

Serotype was not strictly associated with the STs in this MLST study. Several STs were recognized for each serotype except for serotype O2a and O4 in which a unique ST was found. STs associated with sub-founders ST 1 and ST2 were formed by serotype O1a and few O1b strains. Furthermore, serotype O1b strains of *Y. ruckeri* used in this study were recently isolated from previously vaccinated fish (Arias *et al*., 2007; Bastardo *et al*., 2011a). The strong association of sub-founders ST1 and ST2 to the majority of the ERM outbreaks in salmonid cultures also allows to link these STs to the virulence in *Y. ruckeri* strains, and suggest that serotypes O1a and O1b are an example of recently emerged and disseminated variants.

Interestingly, non-motile *Y. ruckeri* strains (biotype 2), causing recent outbreaks in rainbow trout, were included into the sub-founders ST 2 and ST 1 together with other motile strains, indicating that biotype 2 phenotype may have evolved from related motile *Y. ruckeri* strains. These findings are consistent with those reported by Wheeler and colleagues (2009), who using PFGE analysis established that the biotype 2 strains in Denmark and Spain are more closely related to the biotype 1 strain present in these countries than biotype 2 strains from UK. Although the coexistence of non-motile strains from USA, UK, mainland Europe and South America into ST 2 may suggest evidences for cross-border *Y. ruckeri* biotype 2 strains dissemination, the presence of the ST 16 in USA, ST 1 in UK and ST 9, ST 11, ST 12 in Peru, would indicate the independent emergence of biotype 2 in these areas. Similarly to these results, Welch and colleagues (2011) reported that biotype 2 strains from the USA contained the same *fliR* mutant allele found also in UK while differing biotype 2 mutations were detected in strains from mainland Europe. These authors suggested that this mechanism of cross-continent dissemination is unclear and that the distribution of non-motile *Y. ruckeri* strains through Europe is not a simply result of expansion of a single highly successful Biotype 2 clonal group.

In conclusion, our study provides new information on the population structure of *Y. ruckeri* and on the genetic mechanisms behind the emergence of clonal lineages that successfully spread globally. We suggest that the *Y. ruckeri* population structure follows an 'epidemic' model of clonal expansion with well-adapted clones that explode to be widely distributed (Feil *et al*., 2000; Smith *et al*., 2000).

A database from this study was created and was host on publmlst.org (Jolley and Maiden, 2010) and is freely available in Internet (http://pubmlst.org/yruckeri/). This database will be useful in future works studying evolution-

ary and epidemiological relationships between strains and for unambiguous comparison of data generate from laboratories around the world.

Experimental procedures

Bacterial strains

A total of 103 strains of *Y. ruckeri* were tested in this study (Table S1). These strains were temporally (from 1965 to 2009) and geographically (from USA, UK, Portugal, Peru, Finland, Spain, Denmark, Chile, Canada, Germany, Norway and Scotland) diverse, and were isolated from different hosts (salmonid and non salmonid fish and mammals) and environmental sources (water and sediment). All the isolates had been previously confirmed as belonging to *Y. ruckeri* using the specific PCR protocol described by LeJeune and Rurangirwa (2000) or by 16S rRNA gene sequencing (Arias *et al*., 2007; Bastardo *et al*., 2011a,b). The *Y. ruckeri* type strain (NCIMB 2194^T) and reference strains for all recognized serotypes O1a, O1b, O2a, O2b, O3 and O4 were also included (Romalde *et al*., 1993). Bacteria were grown in trypticase soy agar (TSA; Difco) with incubation at 25°C for 48h.

DNA purification and gene amplification and sequencing

Total bacterial DNA was extracted from pure bacterial cultures using the Insta-Gene matrix (Bio-Rad, Madrid, Spain) following the manufacturer's instructions. The DNA concentration was spectrophotometrically quantified and adjusted to of 100 ng μl^{-1}. Purified DNA was maintained at –20°C until use for PCR reactions. Six gene loci were selected for MLST analysis, including *gln*A (glutamine synthetase), *gyr*B (DNA gyrase B subunit), *rec*A (DNA repair and recombination), Y-HSP60 (60-KDs heat shock protein), *dna*J (heat shock protein 40) and *thr*A (aspartokinase-homoserine dehydrogenase). These genes were chosen based on previously published reports of MLST data for *Yersinia* species (Kotetishvili *et al*., 2005; Tinsley *et al*., 2009; Hurst *et al*., 2011). PCR conditions for the six housekeeping gene fragments were as follows: 94°C for 5 min, followed by 35 amplification cycles, each consisting of sequential incubation at 94°C (45 s), 51°C (45 s) and 72°C (1 min), followed by a final incubation at 72°C for 5 min, with exception of *gyr*B for which the annealing temperature was raised to 62°C. The amplified fragments were sequenced in both directions using CEQ 8000 sequencer (Beckman Culter, USA). Sequence data analysis was performed with the DNAstar Seqman program (Lasergene) and the alignments were determined using MEGA version 4.0 (Tamura *et al*., 2007).

Identification of clonal complexes

According to MLST standard (Urwin and Maiden, 2003), the allele types (ATs; particular alleles at particular loci) were distinguished for each strain. The combination of ATs for each isolate defined the sequence type (ST). Relations among the detected STs were analysed using the eBURST v3.0 program (http://eburst.mlst.net) (Feil *et al*., 2004). Two different STs are considered as single-locus variants (SLVs) when they differ from each other at single locus. Double-locus variants (DLVs) are any two STs differing in two loci. The statistical confidences for the ancestral types were assessed using 1000 bootstrap re-samplings. A clonal complex (CC) was composed of at least two STs with SLVs, in which every ST shared at least 5 of the six loci in common with at least one other member of the complex. A singleton was defined as a ST that is not grouped into a CC.

Population genetic analysis

The in-frame sequences at the six loci were concatenated, in the order of loci used to define the allelic profile, to produce a sequence (2786 bp in length) for each strain. Nei's index of diversity (*H*) was calculated for each locus to measure the average gene diversity per locus (Nei, 1978). Number of polymorphic sites, the parameter *Pi* and standardized index of association (I^S_A) were performed using the START2 program available from http://pubmlst.org/software/analysis/start2/. *Pi* denotes the average number of nucleotide differences per site for two randomly selected strains. The statistical I^S_A was tested for the null hypothesis of linkage equilibrium. If there is linkage equilibrium, the expected value of I^S_A is zero; alleles are independently distributed at all loci analysed (Maynard-Smith *et al*., 1993). Estimated recombination rates were derived as described previously by Feil and colleagues (2000), where the per-allele and per-site recombination/mutation (r/m) parameter was calculated empirically. Briefly, any SLV allele differing by one nucleotide and not observed elsewhere in the database as part of another ST was considered to have arisen by mutation. An SLV allele differing by multiples nucleotides or containing a single nucleotide change observed as part of another ST in the database was considered to have originated by recombination.

The minimal number of recombination events R_{min} was computed on biallelic site only by using DnaSP5 software (Rozas, 2009). Split-tree generation for individual loci and concatenated sequence was used for visualizing recombination by mean SplitsTree v 4.0 software (Huson and Bryant, 2006). In addition, a test for selection, the ratio of mean non-synonymous substitutions per non-synonymous site/mean synonymous substitution per syn-

onymous site (d_N/d_S ratio), was calculated by the method of Nei and Gojobori with the jukes-Cantor correction implemented in MEGA 4. This measures the type of selection occurring at each locus. The hypothesis tested is for neutrality ($d_N = d_S$), if $d_N/d_S < 1$, then non-synonymous site is under purifying selection; $d_N/d_S > 1$ indicates positive selection, and $d_N/d_S = 1$ neutrality.

Phylogenetic analysis

PHYLIP software (version 3.69), was employed to construct a phylogenetic tree from the allelic profiles of the 103 *Y. ruckeri* strains by UPGMA. The tree was subsequently displayed by the MEGA version 4.0 (Tamura *et al*., 2007).

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References

Arias, C.R., Olivares-Fuster, O., and Hayden, K. (2007) First report of *Yersinia ruckeri* biotype 2 in the USA. *J Aquat Anim Health* **19:** 35–40.

Austin, B., and Austin, D.A. (2007) *Bacterial Fish Pathogens. Diseases of Farmed and Wild Fish*, 4th edn. Chichester, UK: Praxis Publishing.

Austin, D.A., Robertson, P.A.W., and Austin, B. (2003) Recovery of a new biogroup of *Yersinia ruckeri* from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Syst Appl Microbiol* **26:** 127–131.

Bastardo, A., Bohle, H., Ravelo, C., Toranzo, A.E., and Romalde, J.L. (2011a) Serological and molecular heterogeneity among *Yersinia ruckeri* strains isolated from farmed Atlantic salmon *Salmo salar* in. Chile. *Dis Aquat Organ* **93:** 207–214.

Bastardo, A., Sierralta, V., Leon, J., Ravelo, C., and Romalde, J.L. (2011b) Phenotypical and genetic characterization of *Yersinia ruckeri* strains isolated from recent outbreaks in farmed rainbow trout *Oncorhynchus mykiss* (Walbaum) in Peru. *Aquaculture* **317:** 229–232.

Davies, R.L. (1991) Clonal analysis of *Yersinia ruckeri* based on biotypes, serotypes and outer membrane protein types. *J Fish Dis* **14:** 221–228.

Davies, R.L., and Frerichs, G.N. (1989) Morphological and biochemical differences among isolates of *Yersinia ruckeri* obtained from wide geographical areas. *J Fish Dis* **12:** 357–365.

De Grandis, S.A., and Stevenson, R.M.W. (1982) Variation in plasmid profiles and growth characteristics of *Yersinia ruckeri* strains. *FEMS Microbiol Lett* **15:** 199–202.

Ewing, W.H., Ross, A.J., Brenner, D.J., and Fanning, G.R. (1978) *Yersinia ruckeri* sp. nov., the redmouth (RM) bacterium. *Int J Syst Bacteriol* **28:** 37–44.

Feil, E., Li, B.C., Aanensen, D.M., Hanage, W.P., and Spratt, B.G. (2004) eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol* **186:** 1518–1530.

Feil, E.J., Enright, M.C., and Spratt, B.G. (2000) Estimating the relative contributions of mutation and recombination to clonal diversification: a comparison between *Neisseria meningitidis* and *Streptococcus pneumoniae*. *Res Microbiol* **151:** 465–469.

Fouz, B., Zarza, C., and Amaro, C. (2006) First description of non-motile *Yersinia ruckeri* serovar I strains causing disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum), cultured in Spain. *J Fish Dis* **29:** 339–346.

Gonzalez-Escalona, N., Martinez-Urtaza, J., Romero, J., Espejo, R.T., Jaykus, L.A., and De Paola, A. (2008) Determination of molecular phylogenetics of *Vibrio parahaemolyticus* strains by multilocus sequence typing. *J Bacteriol* **190:** 2831–2840.

Hurst, M.R., Becher, S.A., Young, S.D., Nelson, T.L., and Glare, T.R. (2011) *Yersinia entomophaga* sp. nov., isolated from the New Zealand grass grub *Costelytra zealandica*. *Int J Syst Evol Microbiol* **61:** 844–849.

Huson, D.H., and Bryant, D. (2006) Application of phylogenetic networks in evolutionary studies. *Mol Biol Evol* **23:** 254–267.

Jolley, K.A., and Maiden, M.C.J. (2010) BIGSdb: scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics* **11:** 595.

Kotetishvili, M., Kreger, A., Wauters, G., Morris, J., Sulakvelidze, A., and Stine, C. (2005) Multilocus sequence typing for studying genetic relationships among *Yersinia* species. *J Clin Microbiol* **43:** 2674–2684.

LeJeune, J.T., and Rurangirwa, F.R. (2000) Polymerase chain reaction for definitive identification of *Yersinia ruckeri*. *J Vet Diagn Invest* **12:** 558–561.

Lucangeli, C.S., Morabitoa, S., Capriolia, A., Achenea, L., Busania, L., Mazzolinib, E., *et al*. (2000) Molecular Fingerprinting of strains of *Yersinia ruckeri* serovar O1 and *Photobacterium damsela* subsp. *piscicida* isolated in Italy. *Vet Microbiol* **76:** 273–281.

Maiden, M.C. (2006) Multilocus sequence typing of bacteria. *Annu Rev Microbiol* **60:** 561–588.

Maynard-Smith, J., Smith, N.H., O'Rourke, M., and Spratt, B.G. (1993) How clonal are bacteria? *Proc Natl Acad Sci USA* **90:** 4384–4388.

Miragaia, M.J., Thomas, C., Couto, I., Enright, M.C., and de Lencastre, H. (2007) Inferring a population structure for *Staphylococcus epidermidis* from multilocus sequence typing data. *J Bacteriol* **189:** 2540–2552.

Nei, M. (1978) Estimation of average heterozigosity and genetic distance from a small number of individuals. *Genetics* **89:** 583–590.

Nicolas, P.S., Mondot, S., Achaz, G., Bouchenot, C., Bernardet, J.F., and Duchaud, E. (2008) Population structure of

the fish-pathogenic bacterium *Flavobacterium psychrophilum*. *Appl Environ Microbiol* **74:** 3702–3709.
Perez-Losada, M., Browne, E.B., Madsen, A., Wirth, T., Viscidi, R.P., and Crandall, K.A. (2006) Population genetics of microbial pathogens estimated from multilocus sequence typing (MLST) data. *Infect Genet Evol* **6:** 97–112.
Romalde, J.L., Margariños, B., Barja, J.L., and Toranzo, A.E. (1993) Antigenic and molecular characterization of *Yersinia ruckeri*. Proposal for a new intraspecies classification. *Syst Appl Microbiol* **16:** 411–419.
Ross, A.J., Rucker, R., and Ewing, W.H. (1966) Description of a bacterium associated with redmouth disease of rainbow trout (*Salmo gairdneri*). *Can J Microbiol* **12:** 763–770.
Rozas, J. (2009) DNA sequence polymorphism analysis using DnaSP. *Methods Mol Biol* **537:** 337–350.
Salerno, A., Deletoile, A., Lefevre, M., Ciznar, I., Krovacek, K., Grimont, P., and Brisse, S. (2007) Recombining population structure of *Plesiomonas shigelloides* (*Enterobacteriaceae*) revealed by multilocus sequence typing. *J Bacteriol* **189:** 7808–7818.
Schill, W.B., Phelps, S.R., and Pyle, S.W. (1984) Multilocus electrophoretic assessment of the genetic structure and diversity of *Yersinia ruckeri*. *Appl Environ Microbiol* **48:** 975–979.
Smith, J.M., Smith, N.H., Rourke, M.O., and Spratt, B.C. (1993) How clonal are bacteria? *Proc Natl Acad Sci USA* **90:** 433–4336.
Smith, J.M., Feil, E.J., and Smith, N.H. (2000) Population structure and evolutionary dynamics of pathogenic bacteria. *Bioessay* **22:** 1115–1122.
Stevenson, R.M.W., and Airdrie, D.W. (1984) Serological variation among *Yersinia ruckeri* strains. *J Fish Dis* **7:** 247–254.
Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24:** 1596–1599.
Tinsley, J.W., Barker, M., Priest, F.G., and Austin, B. (2009) Phylogeny and population structure of the fish pathogen *Yersinia ruckeri* as defined by multilocus sequence typing (MLST). *14th International Conference on Diseases of Fish and Shellfish*, p. 340. Eur Ass Fish Pathol, Prague, Czech Republic.
Tinsley, J.W., Austin, D.A., Lyndon, A.R., and Austin, B. (2011) Novel non-motile phenotypes of *Yersinia ruckeri* suggest expansion of the current clonal complex theory. *J Fish Dis* **34:** 311–317.
Urwin, R., and Maiden, M.C. (2003) Multilocus sequence typing: a tool for global epidemiology. *Trends Microbiol* **11:** 479–487.
Verner-Jeffreys, D.W., Haig, S., Welch, T.J., Pond, M.J., Davies, R.L., and Gradnier, R. (2011) Characterization of a serotype O1 *Yersinia ruckeri* isolates from the Isle of Man: further evidence that O antigen serotype is not a reliable indicator of virulence. *Bull Eur Assoc Fish Pathol* **31:** 86–91.
Vos, M., and Didelot, X. (2009) A comparison of homologous recombination rates in bacteria and archaea. *ISME J* **3:** 199–208.
Welch, T.J., Verner-Jeffreys, D.W., Dalsgaard, I., Wiklund, T., Evenhuis, J.P., Garcia Cabrera, J.A., *et al*. (2011) Independent emergence of *Yersinia ruckeri* biotype 2 in the United States and Europe. *Appl Environ Microbiol* **77:** 3493–3499.
Wheeler, R.W., Davies, R.L., Dalsgaard, I., Garcia, J., Welch, T.J., Wagley, S., *et al*. (2009) *Yersinia ruckeri* biotype 2 isolates from mainland Europe and the UK likely represent different clonal groups. *Dis Aquat Organ* **84:** 25–33.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Split decomposition analysis of the concatenated sequences of the six loci for the 30 STs obtained in this study. Split graph was generated by SplitsTree software version 4.0 (Huson and Bryant, 2006).
Fig. S2. A combined split graph of allele for all the six MLST loci. A neighborNet network was constructed from the LogDet distance matrix obtained from the concatenated sequences of the 103 strains, by using with SplitsTree version 4.0 (Huson and Bryant, 2006). Formation of an interconnecting network structure was suggestive of recombination.
Fig. S3. UPGMA tree. Phylogenetic tree was constructed from the allelic profiles of the 103 *Y. ruckeri* strains by the unweighted-pair group method using average linkages (UPGMA) by using PHYLIP version 3.69. Number in the brackets indicated the allele types to the designed ST (in the same order referred in Table 1).
Table S1. Biogeographical information for the *Y. ruckeri* strains studied.

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MULTILOCUS SEQUENCE TYPING (MLST) REVEALS HIGH GENETIC DIVERSITY AND EPIDEMIC POPULATION STRUCTURE FOR THE FISH PATHOGEN *Yersinia ruckeri.*

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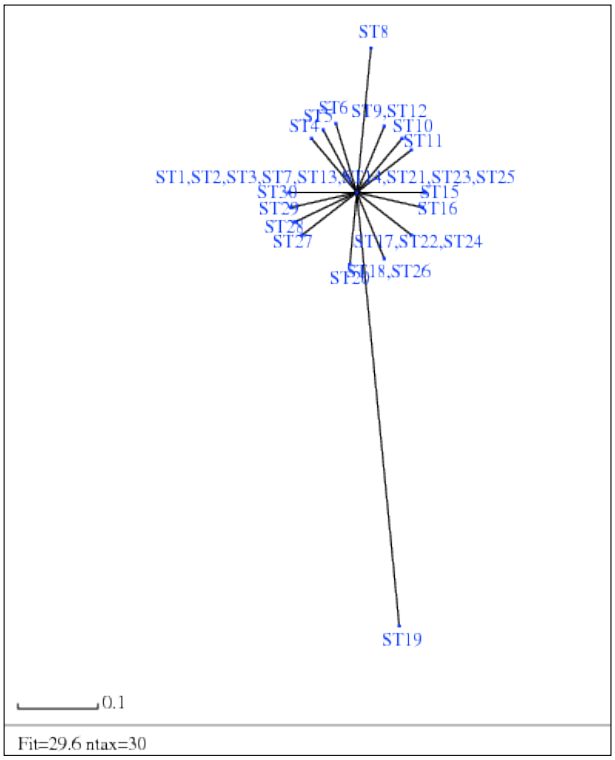
Supplementary Material

Supplementary Table S1. Biogeographical information for the *Y. ruckeri* strains studied.

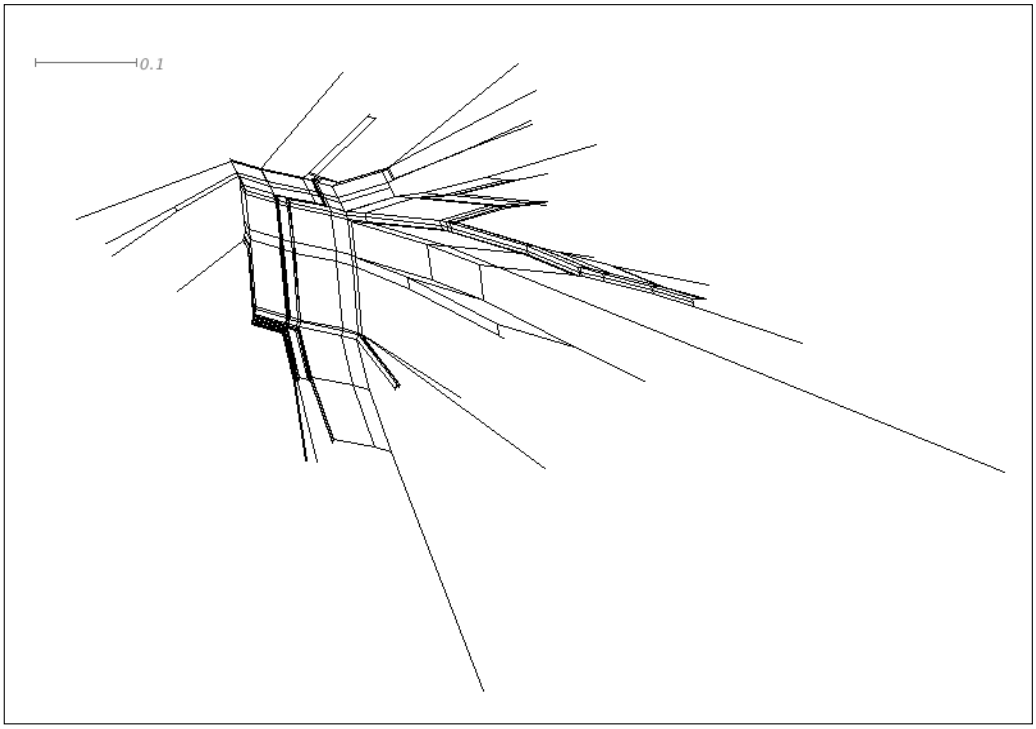
STRAIN	ST	SEROTYPE	BIOTYPE	HOST/SOURCE	ORIGIN	YEAR
NCIMB2194T	1	O1a	1	*Oncorhynchus mykiss*	USA	1965
11.4	2	O1a	1	*O. mykiss*	USA	1979
RS2	5	O2a	1	*Rutilus rutilus*	Canada	1980
RS54	21	O1b	1	*Salmo malma*	Canada	1980
RS76	1	O1a	1	*O. mykiss*	USA	1980
11.47	6	O3	1	*O. mykiss*	USA	1981
11.73	29	O4	1	*O. mykiss*	USA	1981
11.29	4	O2b	1	*O. tshawytcha*	USA	1981
RS75	24	O1	1	*Ondatra zibethica*	Canada	1982
RS80	27	O4	1	*O. mykiss*	USA	1982
820317	25	O1	1	*O. mykiss*	Denmark	1983
830118	23	O1	1	*S. trutta*	Denmark	1983
2/85	20	O2	1	*O. mykiss*	Germany	1985
860821	17	O1	1	*S. salar*	Finland	1985
1533	3	O1b	1	*S. salar*	Norway	1985
850812	22	O2	1	*Anguilla anguilla*	Denmark	1987
860627	1	O1	1	*O. mykiss*	Denmark	1987
AF4	2	O1a	1	*O. mykiss*	Spain	1989
B9-28	19	O1	1	water	Portugal	1994
B16-8	18	O1	1	*O. mykiss*	Portugal	1994
C12-14	3	O3	1	*O. mykiss*	Portugal	1994
C8-3	26	O3	1	*O. mykiss*	Portugal	1994
B14-102	3	O1	1	Sediment	Portugal	1994
C12-54	3	O3	1	Sediment	Portugal	1994
C10-19	26	O3	1	Sediment	Portugal	1994
217/02	23	O2b	1	*O. mykiss*	Spain	2002
37	30	O2b	1	*O. mykiss*	Scotland	2005
LV1	14	O1a	1	*O. mykiss*	USA	2005
SC0314	2	O1b	2	*S. trutta*	USA	2005
SC0415	2	O1b	2	*S. trutta*	USA	2005
SC0309	2	O1b	2	*S. trutta*	USA	2005
SC0415K	2	O1b	2	*S. trutta*	USA	2005
SC03014K	2	O1b	2	*S. trutta*	USA	2005
SC0514	2	O1b	2	*S. trutta*	USA	2005
SC03056	2	O1b	2	*S. trutta*	USA	2005
SC0512	16	O1b	2	*S. trutta*	USA	2005
SC0407	16	O1b	2	*S. trutta*	USA	2005
SC04074	16	O1b	2	*S. trutta*	USA	2005
CA1	2	O1a	2	*O. mykiss*	Portugal	2006
CA2	2	O1a	2	*O. mykiss*	Portugal	2006
CA3	2	O1a	2	*O. mykiss*	Portugal	2006
CA5	2	O1a	2	*O. mykiss*	Portugal	2006
CA6	2	O1a	2	*O. mykiss*	Portugal	2006
CA7	2	O1a	2	*O. mykiss*	Portugal	2006
CA8	2	O1a	2	*O. mykiss*	Portugal	2006
CA9	2	O1a	2	*O. mykiss*	Portugal	2006
CA10	2	O1a	2	*O. mykiss*	Portugal	2006
CA11	2	O1a	2	*O. mykiss*	Portugal	2006
CA12	2	O1a	2	*O. mykiss*	Portugal	2006
CA13	2	O1a	2	*O. mykiss*	Portugal	2006
CA14	2	O1a	2	*O. mykiss*	Portugal	2006
CA15	2	O1a	2	*O. mykiss*	Portugal	2006
BV216	14	O1a	1	*O. mykiss*	UK	2007
E842	13	O1b	1	*O. mykiss*	UK	2007
YR1	1	O1a	2	*O. mykiss*	UK	2001
YR2	1	O1a	2	*O. mykiss*	UK	2001
YR5	1	O1a	2	*O. mykiss*	UK	2001

YR9	2	O1a	2	*O. mykiss*	UK	1995
2576	1	O1a	1	*S. salar*	Chile	2008
2550	1	O1b	1	*S. salar*	Chile	2008
2599	7	O1b	1	*S. salar*	Chile	2008
8386	7	O1b	1	*S. salar*	Chile	2008
8526	7	O1b	1	*S. salar*	Chile	2008
8930	7	O1b	1	*S. salar*	Chile	2008
8958	7	O1b	1	*S. salar*	Chile	2008
8959	7	O1b	1	*S. salar*	Chile	2008
8960	7	O1b	1	*S. salar*	Chile	2008
9394	7	O1b	1	*S. salar*	Chile	2008
6807	8	O2b	1	*S. salar*	Chile	2008
6	1	O1a	1	*O. mykiss*	Peru	2008
7	1	O1a	1	*O. mykiss*	Peru	2008
8	1	O1a	1	*O. mykiss*	Peru	2008
12	1	O1a	1	*O. mykiss*	Peru	2008
16	1	O1a	1	*O. mykiss*	Peru	2008
17	1	O1a	1	*O. mykiss*	Peru	2008
3	2	O1a	1	*O. mykiss*	Peru	2008
10	2	O1a	1	*O. mykiss*	Peru	2008
19	2	O1a	1	*O. mykiss*	Peru	2008
20	2	O1a	1	*O. mykiss*	Peru	2008
23	2	O1a	1	*O. mykiss*	Peru	2008
24	2	O1a	1	*O. mykiss*	Peru	2008
27	2	O1a	1	*O. mykiss*	Peru	2008
28	2	O1a	1	*O. mykiss*	Peru	2008
29	2	O1a	1	*O. mykiss*	Peru	2008
30	2	O1a	1	*O. mykiss*	Peru	2008
A18	2	O1a	1	*O. mykiss*	Peru	2008
A20	2	O1a	1	*O. mykiss*	Peru	2008
A25	2	O1a	1	*O. mykiss*	Peru	2008
9	10	O1a	1	*O. mykiss*	Peru	2008
32	12	O1a	1	*O. mykiss*	Peru	2008
1	28	O1a	1	*O. mykiss*	Peru	2008
13	1	O1a	2	*O. mykiss*	Peru	2008
A24	2	O1a	2	*O. mykiss*	Peru	2008
5	9	O1a	2	*O. mykiss*	Peru	2008
11	9	O1a	2	*O. mykiss*	Peru	2008
15	11	O1a	2	*O. mykiss*	Peru	2008
P20	2	O1a	2	*O. mykiss*	Finland	2009
P21	2	O1a	2	*O. mykiss*	Finland	2009
FP41	2	O1a	1	*O. mykiss*	Spain	1987
AG143	2	O1a	1	*O. mykiss*	Spain	1987
NCTC12269	15	O2b	1		USA	1989
NCIMB1316	2	O1a	1	*S. gairdnieri*	USA	1965
2198-6	1	O1a	2	*O. mykiss*	UK	2006

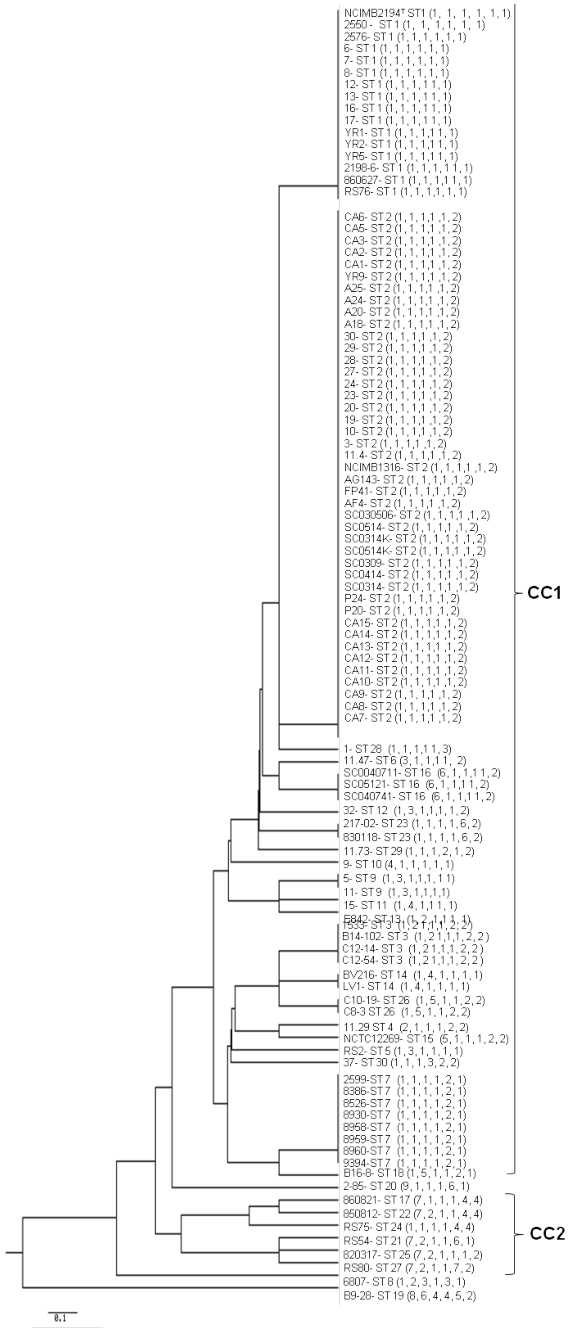
Supplementary Figure S1. Split decomposition analysis of the concatenated sequences of the six loci for the 30 STs obtained in this study. Split graph was generated by SplitsTree software version 4.0 (Huson and Bryant, 2006).



Supplementary Figure S2. A combined split graph of allele for all the six MLST loci. A neighborNet network was constructed from the LogDet distance matrix obtained from the concatenated sequences of the 103 strains, by using with SplitsTree version 4.0 (Huson and Bryant, 2006). Formation of an interconnecting network structure was suggestive of recombination.



Supplementary Figure S3. UPGMA tree. Phylogenetic tree was constructed from the allelic profiles of the 103 *Y. ruckeri* strains by the unweighted-pair group method using average linkages (UPGMA) by using PHYLIP version 3.69. Number in the brackets indicated the allele types to the designed ST (in the same order referred in Table 1).



Artículo:

Phylogeography and demographic history of *Yersinia ruckeri.*

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Resumen:

El patrón filogeográfico y la estructura genética de la población de *Yersinia ruckeri,* el agente patológico de la enfermedad entérica de la boca roja (ERM) en salmónidos, se investigó sobre la base de las secuencias multilócicas concatenadas de cepas con diferentes fenotipos, obtenidas entre 1965-2009 de diversas áreas geográficas y hospedadores. El ánalisis de las secuencias reveló una alta diferenciación genética entre subpoblaciones, observándose mayor distancia genética entre subpoblaciones de Europa y Canadá y/o Suramérica. El análisis Bayesiano indicó la presencia de tres grupos ancestrales dentro de la población. La distribución pareada de frecuencias alélicas revelaron señales características de cambios en la población de *Y. ruckeri,* y también en subpoblaciones geográficamente separadas, debidos a expansiones demográficas y espaciales. Además, se observó un exceso de haplotipos únicos recientes, así como indicios

de aislamiento por distancia. Se observó una correlación positiva significativa entre las distancias genéticas y geográficas. Estos resultados revelaron que la población de *Y. ruckeri* ha experimentado cambios, que fueron inducidos por fuerzas biogeográficas en el pasado y, más recientemente, por procesos de adaptación forzada por la expansión de la acuicultura. Estos hallazgos tienen importantes implicaciones para futuros estudios sobre la dinámica de la población de *Y. ruckeri*, sobre el papel potencial de la estructura genética para explicar las variaciones en la transmisión de la ERM, y sobre el efecto de los eventos evolutivos pasados en las estimaciones actuales del flujo génico.

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Abstract	Phylogeographic patterns and population genetic structure of Yersinia ruckeri, the pathological agent of enteric redmouth disease (ERM) in salmonids, was investigated on the basis of the concatenated multiloci sequences from isolates of different phenotypes obtained between 1965-2009 from diverse areas and hosts. Sequence analyses revealed genetic differentiation among subpopulations with the largest genetic distance occurring between subpopulations of Europe and Canada and/or South America. Bayesian analysis indicated the presence of three ancestral population clusters. Mismatch distribution displayed signatures characteristic of changes in size due to demographic and spatial expansions in the overall Y. ruckeri population, and also in the geographically separate subpopulations. Furthermore, an excess of recent rare haplotypes as well as a weak signal of isolation by distance were determined. A significant positive correlation between genetic and geographical distances was observed. These results revealed that the population of Y. ruckeri has undergone both ancient and recent population changes that were induced by biogeography forces over the past and, much more recently, by adaptive processes forced by aquaculture expansion. These findings have important implications for future studies on Y. ruckeri population dynamics, on the potential role of genetic structure to explain variations in ERM transmission, and on the effect of past evolutionary events on current estimations of gene flow.
Keywords	Phylogeography, Yersinia ruckeri, genetic structure, population changes, aquaculture

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PHYLOGEOGRAPHY AND DEMOGRAPHIC HISTORY OF *Yersinia ruckeri*

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Abstract

Phylogeographic patterns and population genetic structure of *Yersinia ruckeri*, the pathological agent of enteric redmouth disease (ERM) in salmonids, was investigated on the basis of the concatenated multiloci sequences from isolates of different phenotypes obtained between 1965-2009 from diverse areas and hosts. Sequence analyses revealed genetic differentiation among subpopulations with the largest genetic distance occurring between subpopulations of Europe and Canada and/or South America. Bayesian analysis indicated the presence of three ancestral population clusters. Mismatch distribution displayed signatures characteristic of changes in size due to demographic and spatial expansions in the overall *Y. ruckeri* population, and also in the geographically separate subpopulations. Furthermore, an excess of recent rare haplotypes as well as a weak signal of isolation by distance were determined. A significant positive correlation between genetic and geographical distances was observed. These results revealed that the population of *Y. ruckeri* has undergone both ancient and recent population changes that were induced by biogeography forces over the past and, much more recently, by adaptive processes forced by aquaculture expansion. These findings have important implications for future studies on *Y. ruckeri* population dynamics, on the potential role of genetic structure to explain variations in ERM transmission, and on the effect of past evolutionary events on current estimations of gene flow.

Keywords:

Phylogeography/ *Yersinia ruckeri*/genetic structure/population changes/aquaculture.

Introduction

Studies on the evolutionary history of organisms have benefited from the availability of increasing amount of data, especially multiple whole genome sequences. This fact has led to more accurate reconstructions of phylogenetic relationships within several bacterial species (Pritchard et al., 2000). Dispersal, geographic isolation, drift processes and selection leave their signature in the pattern of molecular diversity of contemporary populations (Avise, 2000). Thus, sequence data provide direct genealogical information that can be efficiently used to estimate phylogenetic relationships and parameters associated with population dynamics.

Despite to the limitations of multilocus sequence typing (MLST) and eBURST analysis for the phylogenetic inferences to determine exact relationships between individual isolates, large MLST data represent a valuable resource from which population level trends can be obtained. Analysis of allele frequencies can facilitate recognition of distinct populations, and the comparisons of allelic diversity among populations are informative since ancient populations are expected to be more diverse than recent populations (Slatkin and Hudson, 1991; Gremberghe et al., 2011).

Throughout recorded history, *Yersinia ruckeri*, the etiological agent of enteric red mouth disease (ERM), has been spread multiple times from the USA, probably by egg or carrier fish transfers, as the culture of rainbow trout (*Oncorhynchus mykiss*) became more widely practiced in the world (Austin and Austin, 2007). There is a hypothesis that *Y. ruckeri* could have already existed previously in the USA as suggested by the study of isolates recovered from the National Fisheries Center (USA), which showed to be dated before the first reports of isolation by Rucker in the 1950s (Bullock et al., 1978). In addition, an Australian *Y. ruckeri* isolate was also found dating back in 1960s (Robert, 1983). On the other hand, *Y. ruckeri*

strains in the UK were not reported until 1980s, nevertheless, the first isolations were achieved in the 1970s but the findings were never published (Roberts, 1983).

Although ERM could potentially affect different salmonid fish (mainly to rainbow trout), the microorganism has been isolated from non-salmonid and marine fish (Fuhrmann et al., 1983). Mammals, birds, invertebrates, and even humans were considered as possible vectors of the *Y. ruckeri* (Willumsen, 1989). This bacterium has been also recovered from faeces and sewage sludge and the aquatic environment, including water (Willumson, 1989). In addition, it has been described as a readily biofilm forming bacteria (Coquet et al., 2002). These biofilms may be a source of recurrent infection in rainbow trout farms.

ERM has been successfully controlled for decades by vaccination. Although formulations of most commercial vaccines are based only on common serotype O1a, different degrees of cross-protection among serotypes have been reported (Stevenson, 1997). However, recently ERM vaccine breakdowns have been described in Europe and the USA mostly attributed to *Y. ruckeri* non-motile and lipase negative strains (biotype 2) (Austin et al., 2003; Fouz et al., 2006; Arias et al., 2007). Other epizootics have been reported in Spain, being caused by uncommon serotype O2b in rainbow trout (Romalde et al., 2003), and in Australia and Chile by serotype O1b/biotype 1 strains in vaccinated Atlantic salmon (*Salmo salar*) (Bastardo et al., 2011; Bridle et al., 2012).

Y. ruckeri remains as a concern for aquaculture due to the expanding range of both hosts and pathogen in the world. To understand these processes and to predict future tendencies, detailed data on the contemporary population structure and on the evolutionary and demographic histories that have conformed the populations are essential.

In this study, the genetic structure of a broad geographical range *Y. ruckeri* population was analysed using MLST data to evaluate the evolutionary past of this pathogen within the context of ERM re-emergence. Such data together with information on pathogen-host

associations are critical to understand the dynamics of ERM agent and to form hypothesis concerning its past and future spread.

Materials and methods

Data Set Construction

Data set used in this study consisted of multiloci concatenated sequences of 103 *Y. ruckeri* isolates previously typed into 30 sequence type (ST) and described by an MLST scheme (Bastardo et al., 2012). DNA sequences for 6 housekeeping genes (*gln*A, *gyr*B, Y-HSP60, *rec*A, *dna*J, and *thr*A) were downloaded from the *Yersinia ruckeri* MLST database hosted on publmlst.org and freely available in Internet (http://pubmlst.org/yruckeri/). For analyses, each ST was considered as one haplotype, and the 103 isolates of *Y. ruckeri* were considered as one population, which were divided into nine different subpopulations on the basis of the geographical site of isolation designed in the MLST database (Table 1, Figure 1a).

Phylogenetic and Phylogeographical Analysis

Phylogenetic relationships among the concatenated sequences (2,876 bp) of the 30 haplotypes (equivalent to each ST) were estimated constructing a maximum-likelihood (ML) tree with PHYML 3.0 (Felsenstein, 1989). For the analysis of genetic structure and haplotype sharing, a haplotype network was constructed using the program NETWORK 4.1 (Fluxus-engineering). Concatenated sequences of the six housekeeping genes from all *Y. ruckeri* isolates were previously aligned using the software DNA Alignment 1. 3 (Fluxus-engineering), and the output file were imported into NETWORK 4.1 to conduct a network analysis using a median joining algorithm (Bandelt et al., 1999). The network of closely related haplotypes was displayed using the geographical origins.

Genetic Differentiation

Haplotype polymorphism (Hd), nucleotide diversity (π), and average number of pairwise differences (K) were calculated to assess the genetic variability using the software DnaSP 5 (Librado and Rozas 2009). The pairwise genetic differentiation (F_{ST} index) (Wright, 1965) was determined employing the program Arlequin 3.5 (Excoffier et al., 2010). To assess relatedness among subpopulations, an unweighted pair group method with arithmetic mean (UPGMA) phylogenetic tree inferred from F_{ST} matrix was constructed using the software MEGA 4 (Tamura et al., 2007) and the branch lengths were determined with the program Phylip (Felsenstein, 1989). The F_{ST} matrix was also used to construct a Euclidean-based non-parametric multidimensional scaling (MDS) plot in the software Gedis (Peña et al., 2008). Furthermore, an analysis of molecular variance (AMOVA) was performed to partition genetic variation among regions of restricted gene flow using Arlequin 3.5. The number of migrants N_M (estimated number of migrants between population per generation) was also used to estimate gene flow F_{ST} values.

Genetic Population Structure Analysis

The genetic population structure of *Y. ruckeri* was reconstructed using a Bayesian Monte-Carlo Markov chain (MCMC) sampling method that was implemented using the Structure software (Pritchard et al., 2000). This algorithm identifies genetically distinct populations assigning individual haplotypes to populations on the basis of allele frequencies, and determines the individual membership coefficient in each probabilistic population. For this analysis, an admixture model and the assumption of correlated allele frequencies among subpopulations were assumed (Falush et al., 2003). The probability of assigning individuals into clusters was estimated using 100,000 burn-in repetitions and a final run of one million MCMC steps. The number of clusters (K) was set from 1 to 15, and all runs were replicated

20 times to test the stability of the results. The most probable number of populations (K) was determined by means of the model value (ΔK) based on the second-order rate of change, with respect to K, in the likelihood distribution (Evanno et al., 2005) employing the program Structure harvester (Earl and vonHoldt, 2011).

Spatial Analysis

Geographical coordinates for each isolate were located using Google Earth (htpp://earth.google.com/). The physical distance between different pair of sampling locations was then calculated using the ARC CALC 3 Spherical Trigonometry Calculator macro (http://www.jqjacobs.net/astro/arc_form.html). The presence of phylogeographic structure was tested for individual allele frequencies in six equally-spaced classes of geographic distances by mean the spatial autocorrelation Moran's *I* index using the software Gedis v1.74. Furthermore, to assess the relative influence of drift and gene flow, association between genetic distance and geographic distance was determined employing the Isolation By Distance (IBD) web service v 3.1.6 (Jensen et al., 2005). Significance of the associations was tested with a partial Mantel test using 10,000 randomizations (Mantel, 1967).

Analysis of Demographic History

Historical demographic structure of the genetic variation at the concatenated loci sequences was investigated using coalescent-based Tajima's *D,* Fu and Li *D** and *F**, and Fu's F_S (1997) statistics to test the hypothesis that all mutation are selectively neutral (Tajima, 1989; Fu and Li, 1993). Additional tests of neutrality were also performed by assessing the haplotype structure using Ramos-Onsins' R_2 (Ramos-Onsins and Rozas, 2000) and Strobeck's *S* tests (1987). Analyses of demographic expansion were conducted using DnaSP 5, and the

significance was evaluated by comparing the observed statistics to a distribution of values generated with 5,000 coalescent simulations.

The demographic history of the populations were examined using the frequency of distribution of number of mismatches between pairwise sequences, and by modeling the expected distributions under the demographic scenarios of population constant size using DnaSP 5. The population expansion and spatial expansion assumptions were also modeled employing Arlequin 3.5. The fit model was tested statically by calculating the sum of squared deviation (*SSD*) of the observed data relative to the model.

Harpending's raggedness (*r*) index (Harpending, 1994) was determined to quantify the smoothness of mismatch distributions. Confidence intervals for mismatch distribution parameters were obtained by performing 1,000 bootstrap replicates.

Results

Phylogenetic and phylogeographical relatedness

The median-joining network constructed using all 103 concatenated sequences, illustrated the mutational relationship of the *Y. ruckeri* haplotypes (Figure 1b). All haplotypes (except haplotype 19) differed by less than three mutational steps with a considerable divergence between genomes occurring in different regions. The major groups were separated by one mutational step. Haplotype 2 was the most common interior haplotype found in six different areas, so it is most likely the oldest haplotype. Many haplotypes (13) were tip alleles, being considered as more recently derived and geographically restricted. On the other hand, the majority of haplotypes differed by only one or two mutational steps, suggesting a demographic expansion. Geographical clustering was observed among haplotypes from Portugal (PO), Peru (PE), USA (US) and Chile (CH) subpopulations while the haplotypes present in UK, Denmark/Germany (DG), Finland/Norway (FN), Spain/France (SF) and

Canada (CA) were spread into the network. Phylogenetic relationship among all haplotypes using maximum-likelihood was very poorly resolved and not informative probably because the sequence variation contain insufficient phylogenetic signal (data not shown).

Genetic differentiation

Haplotype diversity ranged from 0.473 to 1.000 among subpopulations with a mean value of 0.792 for the overall population (Table 2). The highest Hd values were observed in CA and DG subpopulations while the lowest value was determined for CH haplotypes. On the other hand, π values ranged to 0.0004 in SF, PE and CH to 0.0017 in CA and DG, regardless the haplotype diversity observed in each area. In general, the average number of pairwise differences (K) within each subpopulation was consistent with Hd values. Furthermore, genetic differentiation based on haplotype diversity among sub-populations was significant (χ^2= 411.87; P< 0.001).

At the finest scale, pairwise F_{ST} values revealed significant differentiation among subpopulations (Table 3). These significant pairwise F_{ST} were detected for PE, CH and US compared with the other subpopulations, and for CA respect to UK. The AMOVA results indicated that the highest proportion of the molecular variance (82.67%) could be explained by variations within each subpopulation, while the rest of the variance is explained by the genetic differences among them (Fisher= 0.1733; P= 0.000). The net number of migrant between subpopulations per generation N_m ranged between 0.5 to infinity (Table 3). Moderate N_m values were found for PE and US respect to the other geographical areas. Only the estimated gene flow for CH was low, indicating that this subpopulation is genetically isolated and/or with limited gene flow. The F_{ST} were used to create a NJ population phylogeny (Figure 2a). Based on an F_{ST} cutoff of 0.05, considered to represent moderate genetic isolation (Wrigth, 1965), three groups can be inferred. Group 1 includes the subpopulations from PE,

US, SF, FN, PO and CA. In addition, it appears to be a secondary division within group 1 including FN, SF, US and PE subpopulations into sub-group 1a, and the CA and PO subpopulations into sub-group 1b. On the other hand, the group 2 included only the DG subpopulation and the group 3 was formed by UK and CH subpopulations. The MDS analysis of all populations recovered similar grouping as showed the NJ tree (Figure 2b).

Genetic population structure

Bayesian analysis of population structure evaluated with Evanno's criterion ΔK, indicated that the data were most consistent with the presence of three genetic populations (K= 3) (Figure 3a). Although ΔK also supported a peak at K= 8, the three major genetic clusters are retained when the K values was increased to 8 (data not shown). The majority of isolates from subpopulations FN, PO, SF, PE, UK, DG, and US appear to share similar proportions of ancestry, with posterior probabilities between 0.500 and 0.798 (Figure 3b). These isolates were included in the genetic cluster I (red colour) with the exception of six isolates from PE, two isolates from FN, and one isolate from UK. These findings evidence the existence of wide spread processes involved in the current distribution of *Y. ruckeri.* On the other hand, all isolates from CH appeared to be distinct, sharing cluster II (green colour) together with other two isolates from PE, and one isolate from UK (posterior probabilities from 0.567 to 0.872) indicating a separate diversification. This observed clustering corroborated in part, the topology showed in the F_{ST}-based NJ tree, and the distribution of populations on the MDS analysis. However, the third genetic cluster (blue colour) included in addition to the DG strains, the rest of isolates from FN, US, and CA excluded in clusters I and II (with posterior probabilities values between 0.567 and 0.872). Interestingly, high levels of admixture were present within all groups.

To check for substructure within the clusters, the analysis was re-run separately for the strains assigned to each cluster. ΔK determined the presence of genetic subpopulations into each cluster establishing K= 5 and K= 6 genetic subpopulations in cluster I (Figure S1a) and cluster II (Figure S1b), respectively. Genetic subpopulations in cluster III were supported by two ΔK peaks at K= 8 and K= 5 but in both scenarios similar proportions of ancestry were retained (Figure S1c). Although relevant geographical differentiations were obtained in concordance with the areas of isolation for the majority of *Y. ruckeri* strains, the isolates found outside of the corresponding genetic subpopulation were indicative of possible migration or introduction processes, gene flow and/or isolation by distance.

An AMOVA conducted by partitioning variation among and within the three clusters revealed that 55.8% of the total variation was attributed to within-population differences (F_{CT}= 0.3504; *P*= 0.000), 31.8% of the variation was associated to the haplotype frequencies among the 3 clusters (F_{SC}= 0.1424; *P*= 0.000), whereas the remaining 9.1% of variation was attributed to within subpopulation-clusters haplotype differences (F_{ST}= 0.4414; *P*= 0.000). Nei's G_{ST} and N_m (1973) were also determined to examine the haplotype pairwise differentiation and gene flow among the clusters (I, II and III), respectively. High level of genetic differentiation (*P*< 0.05) was detected among the three clusters with F_{ST} between 0.0886 and 0.1309, while the estimation of gene flow showed N_m values from 1.9 to 2.3 between populations, supporting limited genetic flow by genetic isolation of clusters.

Demographic history

Tajima's *D* and Fu and Li's *F** and *D** neutrality tests for the overall population analysed had significant negative values (Table 4). These results allowed the rejection of the neutral model in the *Y. ruckeri* population as a result of relatively recent population expansion. Fu's F_S was also negative (F_S= -20.646; *P*< 0.001), which occurs when an excess of rare haplotypes are

present, indicating that population expansion or genetic hitchhiking events. Ramos-Onsins' R_2 statistic and Strobeck's *S* index determined that the total population has significant positive R_2 (0.0560) and high *S* (1.000) values supporting also possible population expansion. However, FN and PO subpopulations showed positive but non-significant F_S values, indicating natural selection or population growth. Positive *D** values exhibited by CA and DG subpopulations suggest balancing selection, although these values were non significant. When neutrality was tested within each genetic cluster (I, II, and III), the results indicated similar conditions to those observed for the overall population (Table 4).

The mismatch distribution of pairwise nucleotide differences in concatenated sequences of the overall *Y. ruckeri* population showed a smooth unimodal distribution, characteristic of a large population expansion (Figure 4a). The *SSD* statistic and raggedness index values were low, supporting these results (Table 5). Study-wide site-frequency spectra revealed an excess of singleton mutation when compared with expected frequencies under a stable population size (Figure 4b). Similar patterns were observed in the individual distribution for each subpopulation except for FN, UK and CA, which exhibited a more ragged distribution (data not shown). FN population differed significantly from the modelled distribution for an expanding population (*P*= 0.04) with high raggedness value (*r*= 0.9722, *P*= 0.05) indicating constant population at equilibrium, although raggedness *P* value supported spatial expansion (*P*= 0.12). Furthermore, the mismatch distribution for each cluster (I, II, and III) also showed a unimodal distribution typical of demographic expansion, supported by the low values for the *SSD* statistic and raggedness index (Table 5).

The mismatch distribution parameter, τ (the time in mutational steps per generation since the modeled expansion event), from the raggedness calculation, was 1.10 (95% CI: 0.65, 1.58) for the demographic expansion, and 1.13 (95% CI: 0.67, 0.12) for the spatial expansion for the entire population of *Y. ruckeri* (Table 5). The divergence times were variable for

subpopulations founding highest estimations in PO and CA for the demographic expansion, and in CH and US for the spatial expansion. On the other hand, for cluster I, τ was estimated at 1.00 (95% CI: 0.00, 53) and 0.54 (95% CI: 0.35, 2.40) mutational steps for both population expansion and spatial expansion, respectively. However, τ parameters were higher for cluster II and cluster III, showing similar τ values for demographic and spatial expansion (τ values between 2.61; 95% CI: 0.86, 6.43 and 4.44; 95% CI: 2.40, 6.69). These findings together provide evidence for demographic and spatial expansion in *Y. ruckeri* population occurring at different times, regardless that the rate of evolution could be similar among subpopulations.

Spatial analysis

Spatial dependence of haplotype frequencies was only detected at the intermediate distance (4,500-6,000 km) (P= 0.000) indicating that the allele frequencies tend to be more different at this geographical distance, and suggesting isolation by distance (Figure 5a). Although not significant Moran's I values were observed within the clusters, this value showed a decrease when the pairwise distances increased (data not shown). The partial Mantel test determined that genetic distances and geographical distances among *Y. ruckeri* subpopulations were positively correlated (Z= 42.10*10^7; r= 0.5915; one-side P= 0.0020) (Figure 5b). Similarly, cluster I showed a positive but non-significant correlation (Z= 24.23* 10^8; r= 0.0260; one-side P= 0.4690). However, clusters II and III provided evidence of non-significant but negative correlation within the subpopulations (cluster II: Z= 14652.4478, r = -0.9532 one sided P= 1.0000, and cluster III: Z= 8189.3193, r = -0.3541 one sided P= 0.8260) supporting the signal of isolation by distance detected for the overall population.

Discussion

In this study, biogeographical and inferred dispersal patterns of *Y. ruckeri* population were analysed on a global scale using multi-sequence data. A pattern of sequence divergence corresponding to geographical area was observed at relatively recent points in the evolutionary history of *Y. ruckeri*. Neighbour network showed a large number of rare haplotypes separated by single-nucleotide differences. Statistical signatures of population expansion were detectable in all subpopulations. Moreover, mismatch distribution indicate that few haplotypes spread and mutated to generate several closely related haplotypes, as is commonly observed during population expansion (Rogers and Harpending, 1992). Furthermore, Tajima's, and Fu and Li tests indicated a significant negative deviation from evolutionary neutrality. If *Y. ruckeri* had emerged in one region early in the past and went on to seed the other regions since them (Austin and Austin, 2007), the same haplotypes in all regions would have predominantly collected and signatures of ancient populations expansions would not be observed. Conversely, these results revealed that *Y. ruckeri* global population had undergone a population expansion some time in the relative recent past.

This study also provided phylogeographical evidence that the ERM disease emergence in South America and Europe over last few decades has been the result of two independent and parallel events. First, the worldwide occurrences of ERM have been facilitated by human-mediated dispersal by transport of fish carrier from North America, and second, intrinsic factors such as genetic differentiation of *Y. ruckeri* population, niche specialization and/or isolation by distance have also played a role. Although trout farming dates back over 400 years in Europe, about 150 years in the USA, and about 100 in South Africa, *Y. ruckeri* was only isolated for the first time from rainbow trout in the Hagerman Valley of Idaho, USA in 1950s (Busch, 1981). Then, the pathogen was increasingly isolated from other states of the USA and from Canada (Wobeser, 1973). The first report of ERM in Europe was published in 1981 by Lesel et al. (1983), whose described the isolation of *Y. ruckeri* from rainbow trout in

the southwestern of France. Subsequently, *Y. ruckeri* have been isolated in the 1980s in Denmark, Italy, Norway, UK, and Spain (Austin and Austin, 2007). In South America, reports about the isolation of *Y. ruckeri* from salmonid are limited. The first occurrences of *Y. ruckeri* in Peru were reported from 1998 to 2000. (Bravo and Kojagura, 2004). In Chile, regardless being one of the largest producers of salmonid, the occurrence of ERM was only reported occurring in Atlantic salmon (*Salmo salar*) in 1992 (Toledo et al., 1993). These reports support the worldwide spreading of *Y. ruckeri* facilitated by the growth and expansion of aquaculture.

Analyses of population structure performed in this study demonstrated a strong genetic separation among *Y. ruckeri* strains in North America, Europe, and South America. The major ancestral population was formed by isolates from USA, Peru, UK, Finland, and Spain, and isolated mainly from *O. mykiss*. The second ancestral population was defined for the majority of isolates from Chile (isolated from *S. salar*, and belonging to O1b serotype) being more closely related to isolates from UK (from *O. mykiss*, serotype O1b), and in less proportion to some isolates from Portugal (isolated from sediment being O3 serotype, and *O. mykiss* O1a). The third ancestral population was formed by *Y. ruckeri* strains from Canada, Portugal, and Denmark not belonging to the serotype O1a, and being the majority of them isolated from hosts different to *O. mykiss*. The evidenced genetic admixture in all the *Y. ruckeri* population supports the previous hypotheses of specificity of *Y. ruckeri* strains and niche specialization (Bastardo et al., 2012).

O. mykiss is native from North American rivers and also from Northern Europe including England, Scotland, Norway, Sweden, Denmark and Germany, as well as France. There are migratory breeds of rainbow trout, which spawn in freshwater and migrate in the same manner as salmon. Furthermore, *O. mykiss* is one of the oldest fish in culture, and the only fish distributed worldwide throughout eyed-eggs transference. Eggs have been stripped since

1872. The first successful transfer of rainbow trout eggs out the North America was made to Japan in 1877. This was followed by shipments to England and Scotland in 1885. European commercial rainbow trout farming started in Denmark in the 1980s, and eggs from hatchery stocks established in England, Scotland and Denmark were transferred to other European countries (Okumus, 2002). This historical spread of *O. mykiss* throughout the world, associated with intensification of aquaculture practices in the last decades, can support the existence of the genetic ancestral populations of *Y. ruckeri* evidenced in this study which may have emerged, coexisted and spread separately, and suggest that the admixture events among them have formed the currently diversified genetic structure in the population of *Y. ruckeri*. The population of *Y. ruckeri* analysed in this study reflected both signatures of population expansion (a non-equilibrium condition) and isolation by distance (which is consistent with evolutionary equilibrium). This phenomenon may be the result of transfer of strains through countries by movement of fish based on aquaculture practices, or by residual effects of range expansion. Humans have had an enormous influence on the global environment and are known to have inflicted genetic variation within other species in the recent past (Conover and Munch, 2002). However, it is still likely that sequences reflect mainly a partial return to evolutionary equilibrium after the expansion event.

Time from population expansion occurred in a population could be estimated based on the parameter τ ($\tau = 2\mu t$, where μ is the mutation rate per nucleotide /year) (Slatkin and Hudson, 1991). Without a known mutation rate from *Y. ruckeri* housekeeping genes, it is not possible to accurately pinpoint the time since the inferred expansion events. However, considering that the typical rates of spontaneous mutation per generation in bacteria have been estimated to be on the order of 10^{-10} substitutions per site per year (Drake, 1991), the mutation rate for *Y. ruckeri* (based on 2,876 bp) can be preliminary estimated to be on the order of 2.8×10^{-7} substitutions per site per year. In theory, if an average of 100-300 generations per year is

assumed (Gordon et al., 2002), the time since expansion indicated by mismatch distribution parameter for the overall population of *Y. ruckeri* is at least several thousand years ago (approximately 6,375-20,000 years), varying between 800 and 30,000 years among the different areas. This fact suggests ancient spread of *Y. ruckeri* long before to the emergence of modern ERM disease, and highlights the scenario of the independent ERM disease emergence events in the North America and Europe in the last centuries. Although the mismatch distributions observed are consistent with expansion in almost all regions, the high values of raggedness index of the Canada, Finland and Portugal subpopulations may indicate a more stable demographic equilibrium with a less sudden expansion (Harpending, 1994).

No evidence for genetic differentiation was obtained in this study to explain the emergence and spread of *Y. ruckeri* biotype 2. However, non-motile isolates were genetically grouped into the same group of motile strains strengthening the theory that biotype 2 has evolved from related motile *Y. ruckeri* strains (Wheeler et al., 2009; Bastardo et al., 2012). The presence of different non-motile haplotypes in the USA, UK, Finland, and Peru support the independent emergence of biotype 2 in geographically separate areas (Ström-Bestor et al., 2010; Bastardo et al., 2012). Furthermore, genetic mixing observed in this study suggests that the emergence of virulent of *Y. ruckeri* variants could be forced by factors extrinsic to the population as resistance to antibiotics or vaccination.

Selective pressure induced by intensive vaccination could cause changes in phenotypic and immunogenic properties, which can cause outbreaks in vaccinated fish. The observed change from *Y. ruckeri* motile to non-motile isolates being recovered from disease outbreaks could be because vaccine induced strain replacement (Martcheva et al., 2008). In this context, Pulkkinen et al. (2010) suggested that the presence of several genetically distinct bacterial populations in one area might favour virulence, if the virulent strains have a competitive advantage. The explanation for the increase of biotype 2 *Y. ruckeri* cases in Europe and USA

as well as the emergence of virulent motile isolates belonging to serotypes O1b and O2b in South America, could be associated with this evolutionary change.

Few studies have addressed questions of phylogeographic structure and dispersal limitation in bacteria on a truly global scale in discontinuos but globally common habitats (Gremberghe et al., 2011). Such studies would provide a realistic insight into the degree of dispersal limitation typically encountered by bacteria. In this study, the significant geographic contribution to the overall genetic differences of *Y. ruckeri* was supported by the positive correlation between genetic and geographic distances among strains and populations. The lack of a total geographic structure could be caused by similar nucleotide diversity across spatial scale among the largest subpopulations of USA, Europe and Peru. Nevertheless, geographic isolation of *Y. ruckeri* haplotypes was evidenced in Chile, Canada, and Denmark, which may have epidemiological implications due to differences in clinical outcomes associated with specific genotypes.

In summary, this study provides phylogeographical findings of signature of ancient demographic processes in *Y. ruckeri* population, including spatial expansion, occurring theoretically at least thousands years ago, and more recent genetic divergence among regions. Furthermore, these results suggest that simultaneous *Y. ruckeri* expansion occurring over last decades are independent, and in some cases isolated events, highlighting the usefulness of genetic studies in explaining the variations in the transmission and maintenance of ERM disease.

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Supplementary information

Supplementary Figure S1 (.doc) is available at ISME Journal
http://www.nature.com/ismej/index.html

References

Arias CR, Olivares-Fuster O, Hayden K, Shoemaker CA, Grizzle JM, Klesius PH. (2007). First report of *Yersinia ruckeri* biotype 2 in the USA. J Aquat Anim Health 19:35-40.

Austin B, Austin DA. (2007). Bacterial Fish Pathogens: Disease in Farmed and Wild Fish (4th ed). Springer Praxis: Press Chichester.

Austin DA, Robertson PAW, Austin B. (2003). Recovery of a new biogroup of *Yersinia ruckeri* from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum) Syst Appl Microbiol 26:127-131.

Avise J. (2000). Phylogeography: the history and formation of species. Cambridge, Harvard University Press: Massachussets.

Bandelt H-J, Forster P, Röhl A. (1999). Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16:37-48.

Bastardo A, Bohle H, Ravelo C, Toranzo AE, Romalde JL. (2011). Serological and molecular heterogeneity among *Yersinia ruckeri* strains isolated from farmed Atlantic salmon *Salmo salar* in Chile. Dis Aquat Org 93:207-214.

Bastardo A, Ravelo C, Romalde JL. (2012). Multilocus sequence typing reveals high genetic diversity and epidemic population structure for the fish pathogen *Yersinia ruckeri*. Environ Microbiol 14:1888-1897.

Bravo S, Kojagura V. (2004). First isolation of *Yersinia ruckeri* from rainbow trout (*Oncorhynchus mykiss*) in Peru. Bull Eur Ass Fish Pathol 24:104-108.

Bridle AR, Koop BF, Nowak BF. (2012). Identification of surrogates of protection against yersiniosis in immersion vaccinated atlantic salmon. PLoS One 7:e40841.

Bullock G, Stuckkey H, Shotts E. (1978). Enteric redmouth bacterium: comparison of isolates from different geographic areas. J Fish Dis 1:351-356.

Busch RA. (1981). Enteric redmouth disease (*Yersinia ruckeri*). In: Anderson DP, Dorson M, Dubourget P. (eds). Antigens of fish pathogens. development and production of vaccines and serodiagnostics. Collection Foundation Marcel Merieux: Lyon, pp 201-226.

Conover DO. Munch SB. (2002). Sustaining fisheries yields over evolutionary time scales. Science. 297:94-96.

Coquet L, Cosette P, Junter GA, Beucher E, Saiter JM, Jouenne T. (2002). Adhesion of *Yersinia ruckeri* to fish farm materials: influence of cell and material surface properties. Colloid Surf B 26:373-378.

Drake JW. (1991). A constant rate of spontaneous mutation in DNA-based microbes. Proc Natl Acad Sci 88:7160-7164.

Earl DA, vonHoldt BM. (2011). Structure Harvester: a website and program for visualizing structure output and implementing the Evanno method. Conserv Genet Resour 4:359-360.

Evanno G, Regnaut S, Goudet J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14:2611-2620.

Excoffier L, Lischer HEL. (2010). Arlequin suite v 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour 10:564-567.

Felsenstein J. (1989). PHYLIP phylogeny inference package (Version 3.2). Cladistics 5:164-166.

Fouz B, Zarza C, Amaro C. (2006). First description of non-motile *Yersinia ruckeri* serovar I strains causing disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum), cultured in Spain. J Fish Dis 29:339-346.

Fu Y-X, Li W-H. (1993). Statistical tests of neutrality of mutations. Genetics 133:693-709.

Fu Y.X. (1997). Statistical test of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147:915-925.

Fuhrmann H, Bohm KH, Schlotfeldt HJ. (1983). An outbreak of enteric redmouth disease in West Germany. J Fish Dis 6:309-311.

Gordon DM, Bauer S, Johnson JR. (2002). The genetic structure of Escherichia coli populations in primary and secondary hábitats. Microbiology 148:1513-1522.

Harpending HC. (1994). Signature of ancient population growth in a low-resolution mitocondrial DNA mismatch distribution. Hum Biol 66:591-600.

Jensen JL, Bohonak AJ, Kelley ST. (2005). Isolation by distance, web service. BMC Genetics 6:13.

Lesel R, Lesel M, Gavini F, Vuillaume A. (1983). Outbreak of enteric redmouth disease in rainbow trout, *Salmo gairdneri* Richardson, in France. J Fish Dis 6:385-387.

Librado P, Rozas J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451-1452.

Mantel N. (1967). The detection of disease clustering and a generalized regression approach. Cancer Res 27:209-220.

Martcheva M, Bolker BM, Holt RD. (2008). Vaccine-induced pathogen strain replacement: What are the mechanisms? J R Soc Interface 5:3-13.

Moran PAP. (1950). Notes on continuous stochastic phenomena. Biometrika 37:17-23.

Okumus I. (2002). Rainbow trout broodstock management and production in Turkey: present practices, constraints and the future. Turk J Fish Aquat Sci 2:41-56.

Peña JA, Alfonso-Sánchez MA, Pérez-Miranda AM, García-Obregón S, Gómez-Pérez L. (2009). GeDis: Un programa para análisis de datos en Antropogenética. Antropo 20:49-56.

Pritchard JK, Stephens M, Donnelly P. (2000). Inference of population structure using multilocus genotype data. Genetics 155:945-959.

Pulkkinen K, Suomalainen L-R, Read AF, Ebert D, Rintamäki P, Valtonen ET. (2010). Intensive fish farming and the evolution of pathogen virulence: the case of columnaris disease in Finland. Proc R Soc B 277:593-600.

Ramos-Onsins SE, Rozas J. (2000). Statistical properties of new neutrality tests against population growth. Mol Biol Evol 19:2092-2100.

Roberts MS. (1983). A report of an epizootic in hatchery reared rainbow-trout, *Salmo gairdneri* Richardson, at an English trout farm, caused by *Yersinia-ruckeri*. J. Fish Dis. 6:551-552.

Rogers AR, Harpending H. (1992). Population growth makes waves in the distribution of pairwise genetic differences. Mol Biol Evol 9:552-569.

Romalde JL, Planas E, Sotelo JM, Toranzo AE. (2003). First description of *Yersinia ruckeri* serotype O2 in Spain. Bull Eur Assoc Fish Pathol 23:135-138.

Slatkin M, Hudson RR. (1991). Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. Genetics 129:555-562.

Stevenson RMW. (1997). Immunization with bacterial antigens: Yersiniosis. Dev Biol Stand 90:117-124.

Strobeck C. (1987). Average number of nucleotide differences in a sample from a single subpopulation: a test for population subdivision. Genetics 117:149-153.

Ström-Bestor M, Mustamäki N, Heinikainen S, Hirvelä-koski V, Verner-Jeffreys D, Wiklund T. (2010). Introduction of *Yersinia ruckeri* biotype 2 into Finnish fish farms. Aquaculture 308:1-5.

Tajima F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585-595.

Tamura K, Dudley J, Nei M, Kumar S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596-1599.

Toledo MS, Troncoso M, Portell DP, Figueroa G. (1993). Outbreak due to *Yersinia ruckeri* in cultured salmonids. Ann Microbiol 1:59-62.

van Gremberghe I, Leliaert F, Mergeay J, Vanormelingen P, Van der Gucht K, Debeer A, Lacerot G, De Meester L, Vyverman W. (2011). Lack of phylogeographic structure in the freshwater cyanobacterium *Microcystis aeruginosa* suggest global dispersal. PLoS One 6:e19561.

Wheeler RW, Davies RL, Dalsgaard I, Garcia J, Welch TJ, Wagley S, Bateman KS, Verner-Jeffreys DW. (2009). *Yersinia ruckeri* biotype 2 isolates from mainland Europe and the UK likely represent different clonal groups. Dis Aquat Organism 84:25-33.

Willumsen B. (1989). Birds and wild fish as potential vectors of *Yersinia ruckeri.* J Fish Dis 12:275-277.

Wobeser G. (1973). An outbreak of redmouth disease in rainbow trout (*Salmo gairdneri*) in Saskatchewan. J Fish Res Board Can 30:571-575.

Wright S. (1965). The interpretation of population structure by F-statistics with special regard to system of mating. Evolution 19:395-420.

560 Table 1. Summary information of *Y. ruckeri* isolates used in this study.

Location id	Geographical origin	MLST type[1]	Biotype	Serotype	Host or Source	Year of isolation
CA	Canada (5)	ST5, ST15, ST21, ST24, ST25	BT1	O1a, O1b, O2a, O2b, O4	*O. mykiss* (3) *O. Zibethica* *S. malma*	1965/ 1980
FN	Finland (3) Norway (1)	ST 2 (2), ST 3, ST 17	BT1 (2) BT2 (2)	O1a (3), O1b	*O. mykiss* (2) *S. salar* (2)	1985/ 2009
DG	Denmark (4) Germany (1)	ST1, ST20, ST22, ST23, ST25	BT1 (5)	O1a (2), O2a (1), O2b (2)	*O. mykiss*, (2) *S. trutta* *A. anguilla*	1983/ 1989
UK	United Kingdom (8)	ST1 (4), ST2 (1), ST13, ST14, ST30	BT1 (3) BT2 (5)	O1a (6), O1b, O2b	*O. mykiss* (8)	1995/ 2007
PO	Portugal (21)	ST2 (14), ST3 (3), ST8, ST19, ST26 (2)	BT1 (7) BT2 (14)	O1a (17) O3 (4)	*O. mykiss* (17) sediment (3) water	1994/ 2006
SF	Spain (4) France (1)	ST2 (4), ST23	BT1	O1a (4), O2b	*O. mykiss*	1980/ 2002
PE	Peru (27)	ST1 (7), ST2 (14), ST9 (2), ST10, ST11, ST12, ST28	BT1 (22) BT2 (5)	O1a	*O. mykiss*	2008
CH	Chile (11)	ST1 (2), ST7 (8), ST8	BT1	O1a (2), O1b (8), O2b	*S. salar*	2008
US	United State (17)	ST1, ST2 (10), ST4, ST6, ST14, ST16 (3),	BT1 (7) BT2 (10)	O1a (4), O1b (10), O2b, O3, O4	*O. mykiss* (6) *S. trutta* (10) *O. tshawytshcha* (1)	1965/ 2006

[1]Established by Bastardo et al. (2012). Each ST corresponds to one haplotype in this study.

Table 2. Statistics of genetic variation observed within the nine populations of *Y. ruckeri.*

Location id	N	*K*	Hd	π
CA	5	4.8000±2.8173	1.000±0.126	0.0017±0.0011
FN	3	3.0000±1.9639	0.833±0.222	0.0011±0.0008
DG	5	4.8000±2.8173	1.000±0.126	0.0017±0.0011
UK	5	1.9642±1.2384	0.785±0.150	0.0007±0.0005
PO	5	4.3809±2.2536	0.548±0.118	0.0016±0.0009
SF	3	1.2000±0.9084	0.700±0.218	0.0004±0.0003
PE	7	1.0598±0.7241	0.656±0.085	0.0004±0.0002
CH	3	1.0546±0.7548	0.473±0.161	0.0004±0.0003
US	7	1.3529±0.8797	0.713±0.109	0.0005±0.0003
Overall	30	2.6712±0.890	0.792±0.037	0.0010±0.0002

N, number of haplotypes; *K*, average number of pairwise differences; Hd, haplotypes diversity; π, nucleotide diversity. Location id as shown in Table 1.

Table 3. Matrix of pairwise genetic differentiation (F_{ST}), and net number of migrants (N_m) among different sub-population of *Yersinia ruckeri*.

	CA	**FN**	**DG**	**UK**	**PO**	**SF**	**PE**	**CH**	**US**
CA	-	∞[1]	∞	1.9	12.6	3.1	0.6	0.7	1.1
FN	-0.1422[2]	-	∞	5.3	∞	∞	1.7	0.6	4.5
DG	-0.1215	-0.1262	-	∞	27.5	12.5	1.1	1.2	1.3
UK	0.2126*	0.0860	0.0652	-	9.4	72.7	6.3	2.0	2.1
PO	0.0381	-0.0938	0.0179	0.0507	-	∞	3.2	1.4	5.7
SF	0.1379	-0.0253	0.0385	0.0068	-0.0319	-	23.8	0.5	104.6
PE	0.4454*	0.2264*	0.3199*	0.0729	0.1356*	0.0201	-	0.5	2.7
CH	0.4212*	0.4677*	0.2913*	0.2001*	0.2538*	0.4606*	0.4891*	-	0.4
US	0.3187*	0.1006	0.2761*	0.1938*	0.0801*	0.0047	0.1552*	0.5354*	-

[1]Upper corner: N_m (number of migrants per generation) estimated from F_{ST} values. [2]Lower corner: Pairwise F_{ST} values. * Shows a significant *P*-value (<0.05). Location id as shown in Table 1.

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Table 4. Results of neutrality tests for different locations *Y. ruckeri* studied

Location	**N**	***D***	R_2	F_S	***S***	***D****	***F****
Overall	103	-2.2864**	0.0560*	-20.646**	1.000	-6.1456**	-5.4848**
CA	5	0.7896	0.1315	-1.411	1.000	0.2981	0.2516
FN	4	-0.8086	0.4714	0.731	0.775	-0.8086	-0.7772
DG	5	0.7896	0.1957	-1.411	1.000	0.7896	0.8301
UK	8	-0.7046	0.1916	-1.191	0.936	-0.7219	-0.7922
PO	21	-2.0377*	0.1751	3.528	0.089	-3.3770**	-3.4791**
SF	5	-1.0485	0.4330	-0.186	0.874	-1.0485	-1.0519
PE	27	-0.9354	0.0989	-2.719	0.981	-1.8029	-1.7990
CH	11	-1.4646	0.2323	0.694	0.652	-1.4445	-1.6340
US	17	-1.1974	0.1002	-2.838	0.985	-1.0739	-1.2746
Cluster 1	73	-1.7293*	0.0431	-9.6119**	1.000	-3.4612**	-3.4509**
Cluster II	14	-0.6503	0.1223	-1.2323	0.916	-0.6503	-0.9652
Cluster III	16	-1.6183*	0.1712	-3.9302**	0.996	-2.4651*	-2.5921*

N, number of sequences analyzed; *D*, Tajima's index; R_2, Ramos-Onsins' test; F_S, Fu's statistic; * D* and *F**, Fu and Li tests; *S*, Strobeck's statistic.*, significance at $P< 0.05$; ** shows significance for Fu's and for Fu and Li tests at $P <0.001$, and for Tajima's index at $P< 0.01$, respectively. Location id as shown in Table 1.

605 Table 5. Results of Mismatch distribution analyses for different locations *Y ruckeri* studied.

Location id	N	*SSD*[†]	*SSD*[‡]	*r*	τ^1	τ^2
Overall	103	0.0802	0.0068	0.0068	1.10 (0.65-1.58)	1.13 (0.67-0.12)
CA	5	0.0870	0.0870	0.2800	4.93 (1.56-7.73)	4.93 (1.27-7.45)
FN	4	0.3278*	0.3137*	0.9722*[1]	3.45 (0.89-6.11)	3.47 (0.67-69)
DG	5	0.0393	0.0393	0.1200	2.74 (0.77-7.98)	2.73 (0.91-8.63)
UK	8	0.0307	0.0280	0.1300	2.51 (0.31-5.36)	2.22 (0.69-5.35)
PO	21	0.0869	0.0236	0.2291	5.19 (0.42-91.19)	3.67 (0.00-9.28)
SF	5	0.0062	0.0052	0.0050	1.57 (0.00-2.91)	1.52 (0.00-3.37)
PE	27	0.0011	0.0010	0.0471	1.08 (0.42-1.87)	0.82 (0.28-2.23)
CH	11	0.0319	0.0295	0.1743	0.15 (0.00-1.58)	4.69 (0.00-124.0)
US	17	0.0132	0.0138	0.1074	0.15 (0.00-1.59)	4.69 (0.00-124.0)
Cluster 1	73	0.0019	0.0013	0.0557	1.00 (0.00-53.5)	0.54 (0.35-2.40)
Cluster II	14	0.0464	0.0355	0.1331	3.89 (0.00-6.416)	2.61 (0.86-6.43)
Cluster III	16	0.0091	0.0092	0.0238	4.44 (2.40- 6.69)	4.12 (1.89-5.73)

N, number of sequences analyzed; *r*, *SSD*, mismatch distribution; Raggedness index; τ, divergence time; [1]under demographic expansion model; [2]under spatial expansion model; *, significance at $P < 0.05$. Location id as shown in Table 1.

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Titles and legends to figure

Figure 1. Map of study area showing the geographic location of *Yersinia ruckeri* isolates (a), and haplotype network of *Yersinia ruckeri* (b). Colours indicate the different geographic area as in Table 1. Circles represent each haplotype. A line between haplotypes represent one mutational step, open circles represents haplotypes not present in the sample. Radius of the circle represents is proportional to the number of sequences.

Figure 2. Clustering analysis. a) F_{ST} distanced-based neighbor-joining tree inferred for *Yersinia ruckeri* subpopulations. F_{ST} values are proportional to the branch lengths. Brackets indicate possible genetic clustering of 3 groups (1, 2, and 3). Distance between group 1 and group 2, F_{ST}= 0.0789; between group 2 and group 3, F_{ST}= 0.0824; between group 1 and group 3, F_{ST}= 0.0927. Within group 1, subgroup 1a, and subgroup 1b are also indicated. b) Non-metric multi-dimensional scaling (MDS) of *Y. ruckeri* populations based on pairwise F_{ST} distances. MDS provide a visual representation of the pattern of distance proximities among different populations. Shepard's diagram shows the reproduced distances plotted on the vertical (*Y*) axis versus the original similarities plotted on the horizontal (*X*) axis. Stress measures of goodness-of-fit; Strees <0.15 are considered acceptable.

Figure 3. Structure analysis of 103 *Yersinia ruckeri* strains at optimal K= 3 value. Each vertical bar represents a single isolate. The height of each colour represents the probability of assignment to that cluster. Subpopulations are listed at the top and their geographical origins as indicated in Table 1. For each plotting strains ordered by ancestry coefficients (a) and by geographic origin (b) are showed.

Figure 4. Population expansion signals in *Yersinia ruckeri* sequence data. a) Site-frequency spectrum indicating excess of singleton mutations in sequences. Spectrum compares observed frequencies of segregating sites to expected distribution under the null hypothesis of no population change. b) Mismatch distribution of observed frequencies of pairwise difference among concatenates sequences and expected frequencies, under neutral model of evolution given the null hypothesis of no population change, population expansion, and spatial expansion.

Figure 5. Spatial analysis. a) Moran's correlogram of individual allele frequencies. Moran's *I* was plotted for individual allele frequencies across 6 distance classes (line black). Significant value (dot black) of Moran's *I* indicate positive spatial dependence at $P< 0.05$. b) Mantel test for isolation by distance. Regresion based on genetic distance ($PhiF_{ST}$) values among 9 subpopulations. Regresion slope= 0.0431 ± 0.005; $R^2 = 0.350$ Mantel probability $P< 0.01$.

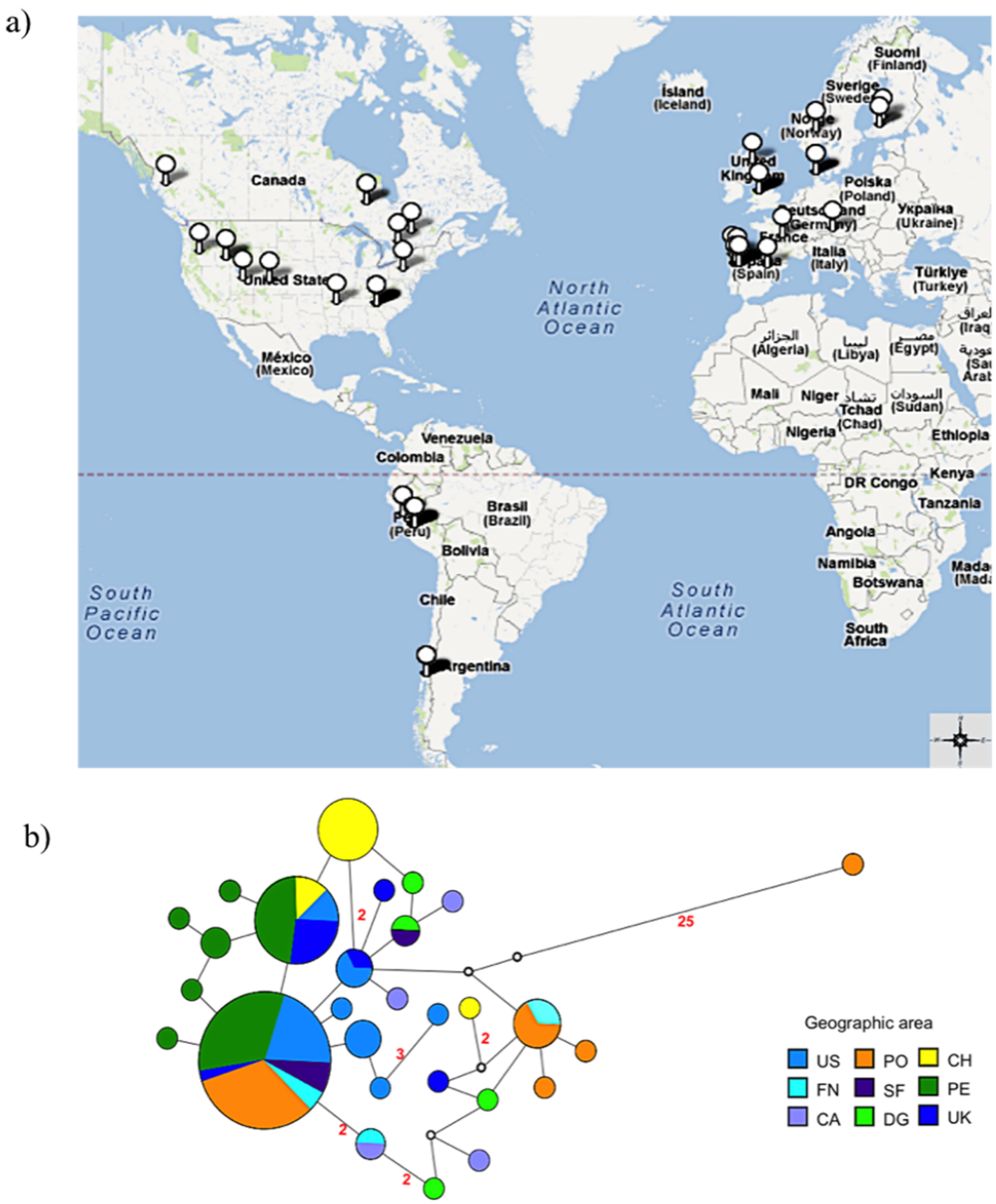


Figure 1. Bastardo et al.

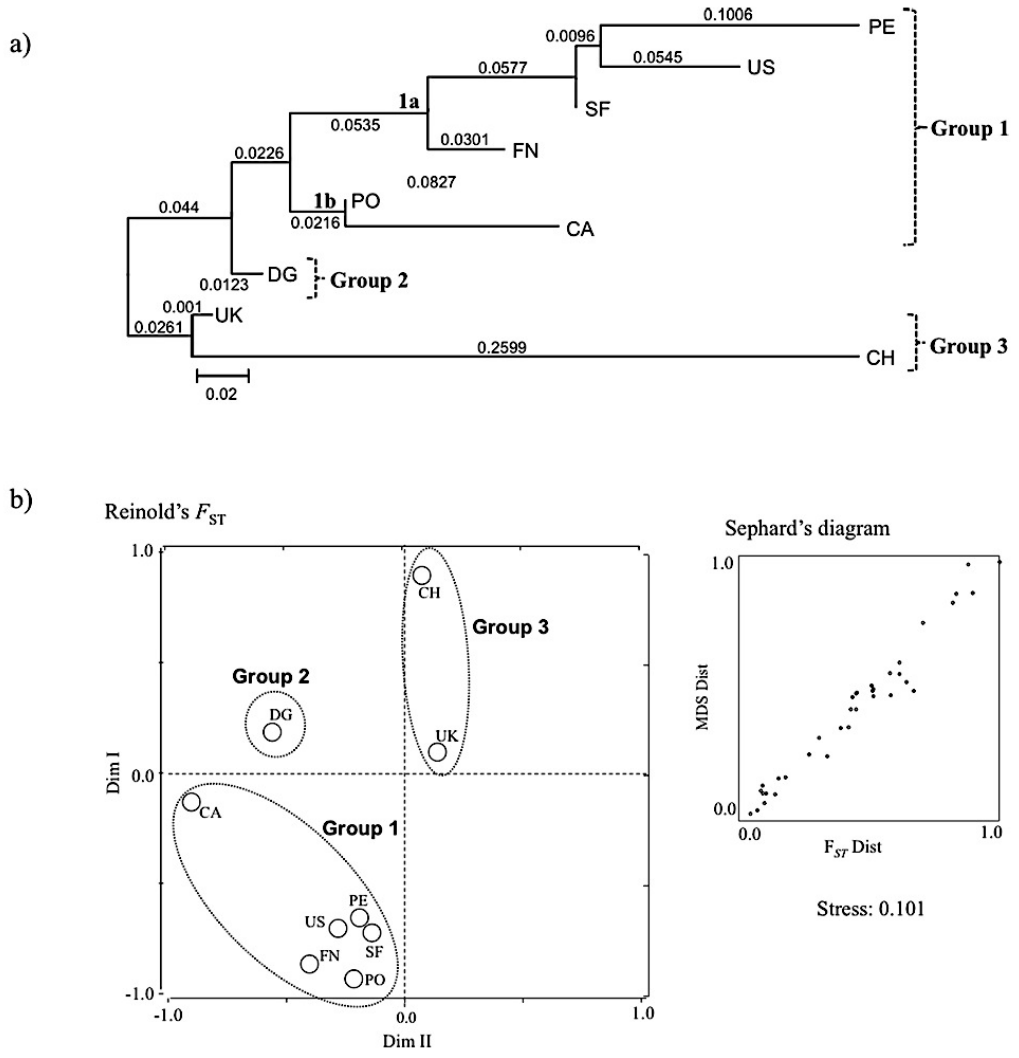


Figure 2. Bastardo et al.

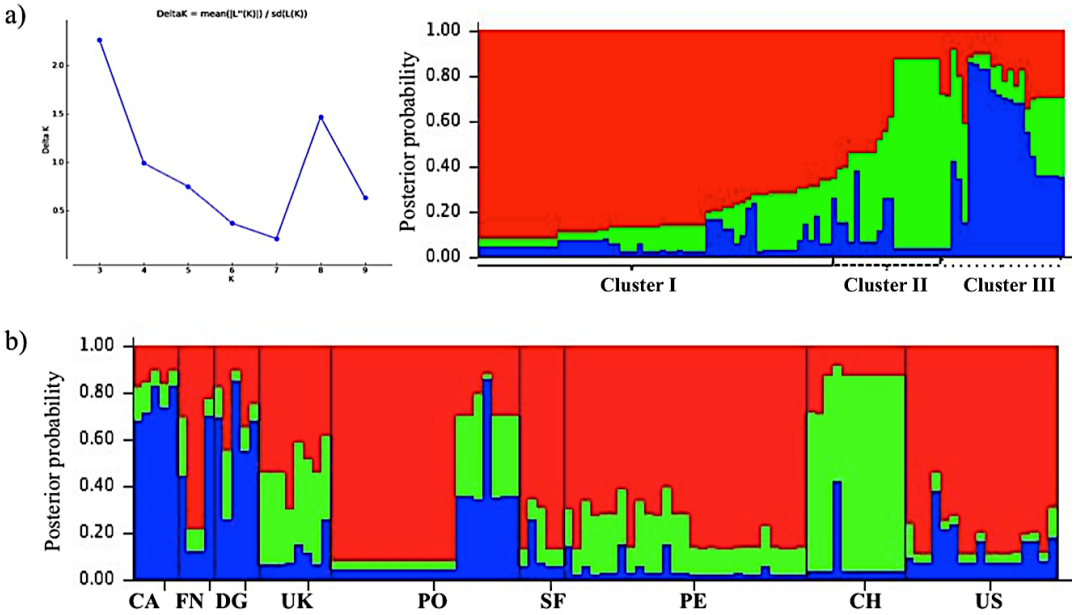


Figure 3. Bastardo et al.

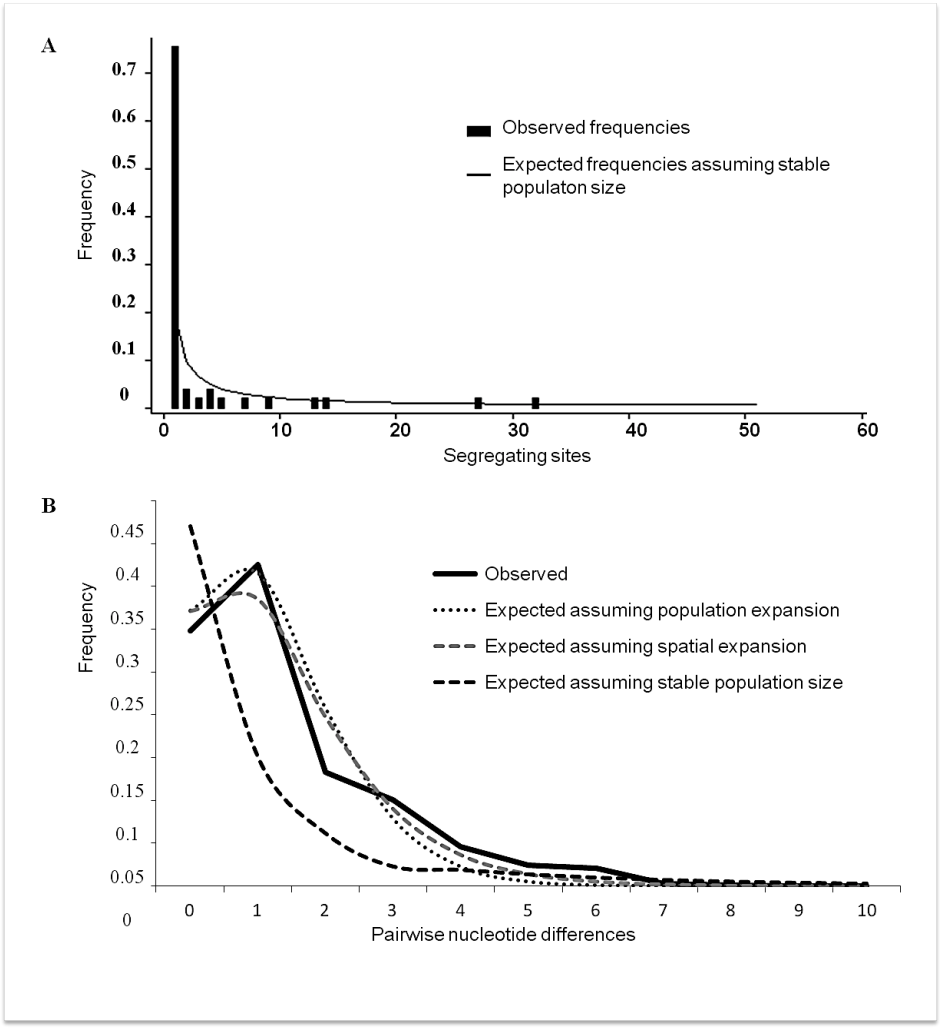


Figure 4. Bastardo et al.

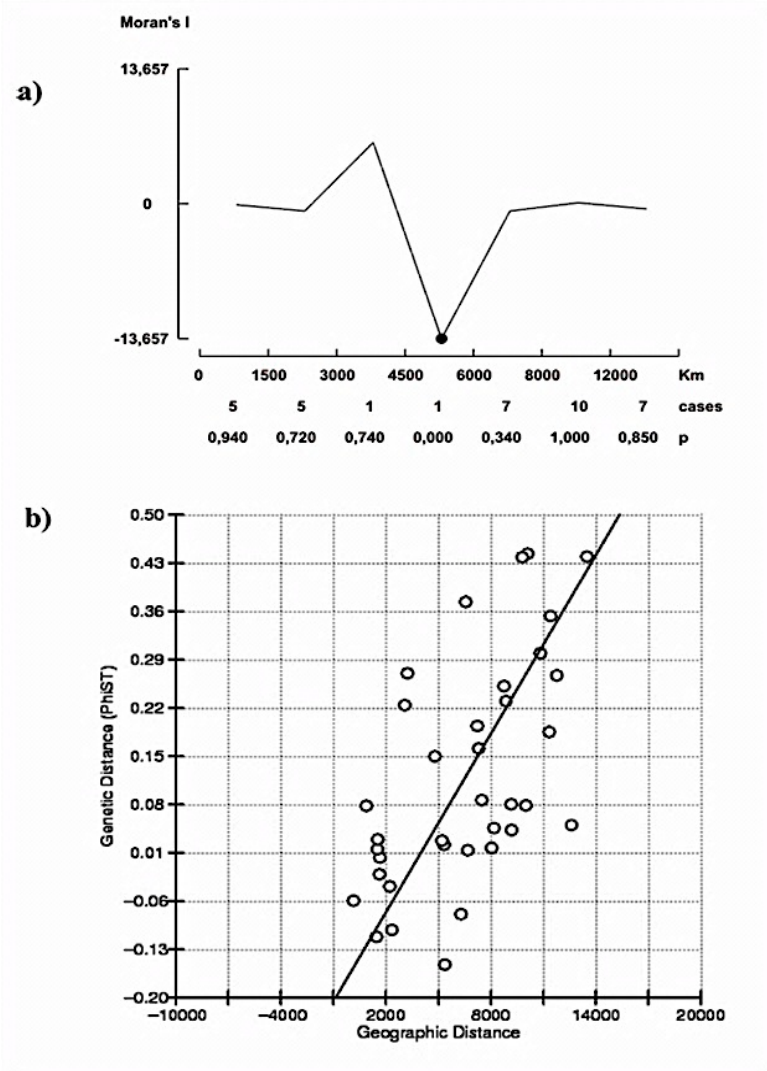


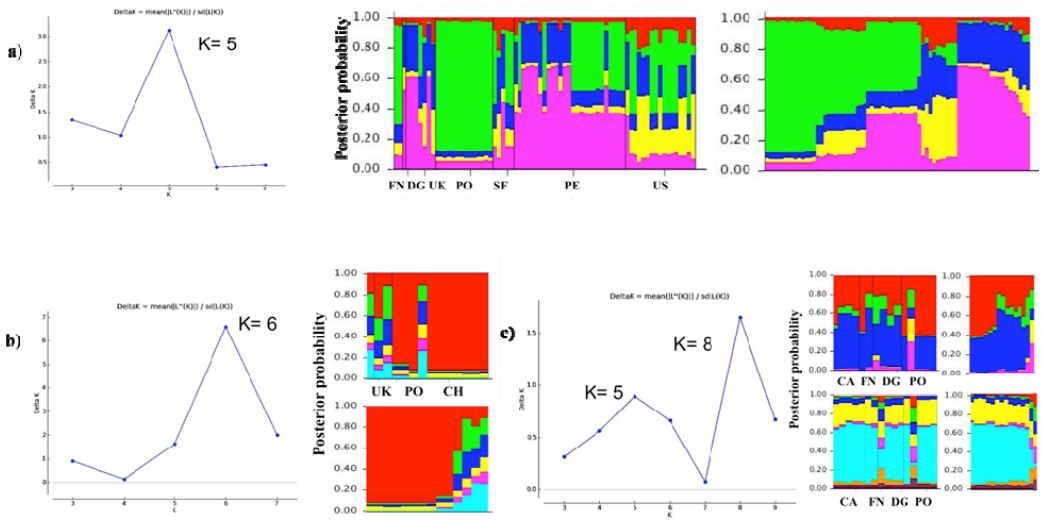
Figure 5. Bastardo et al.

Phylogeography and demographic history of *Yersinia ruckeri*

Asmine Bastardo, Carmen Ravelo, Jesús L. Romalde

Supplementary information

Figure S1. Populations and subpopulations inferred in clusters I, II, and III by STRUCTURE analyses. The peak at ΔK represents the most probable number of populations and subpopulations. a) K= 5 subpopulations identified within cluster I. b) K= 6 subpopulations identified within cluster II, and c) K= 8 and K= 5 subpopulations determined within the cluster III. Each vertical line in the structure bar plot represents each strain. Different colored segments on the vertical bar represent strains with mixed membership coefficient (maximum 1) to the different population, subpopulation or cluster. For each cluster, plotting ordered by geographic origin, and ancestry coefficients are showed.



FIGURAS COMPLEMENTARIAS CAPÍTULO II.

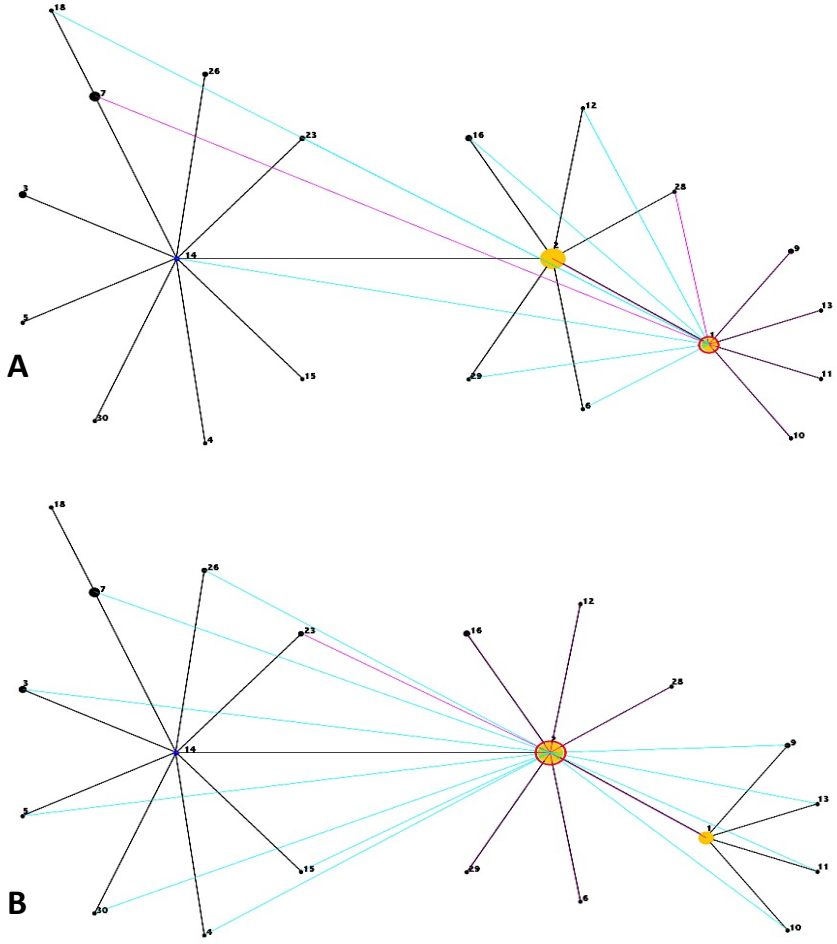


Figura complementaria 1. Complejo clonal 1 determinado por el análisis eBURST para *Yersinia ruckeri* en base al esquema de tipado de sequencias multilócicas (MLST). Los tipos de secuencias (ST) que se diferencian en un solo locus (SLV) se muestran conectados por líneas negras y/o rosa. Los STs que se diferencian en dos locus (DLV) se muestran conectados por líneas azules. El ST ancestral y los sub ancestrales se representan con un círculo azul y amarillo respectivamente. A) SLVs y DLVs para el ST 1. B) SLVs y DLVs para el ST 2.

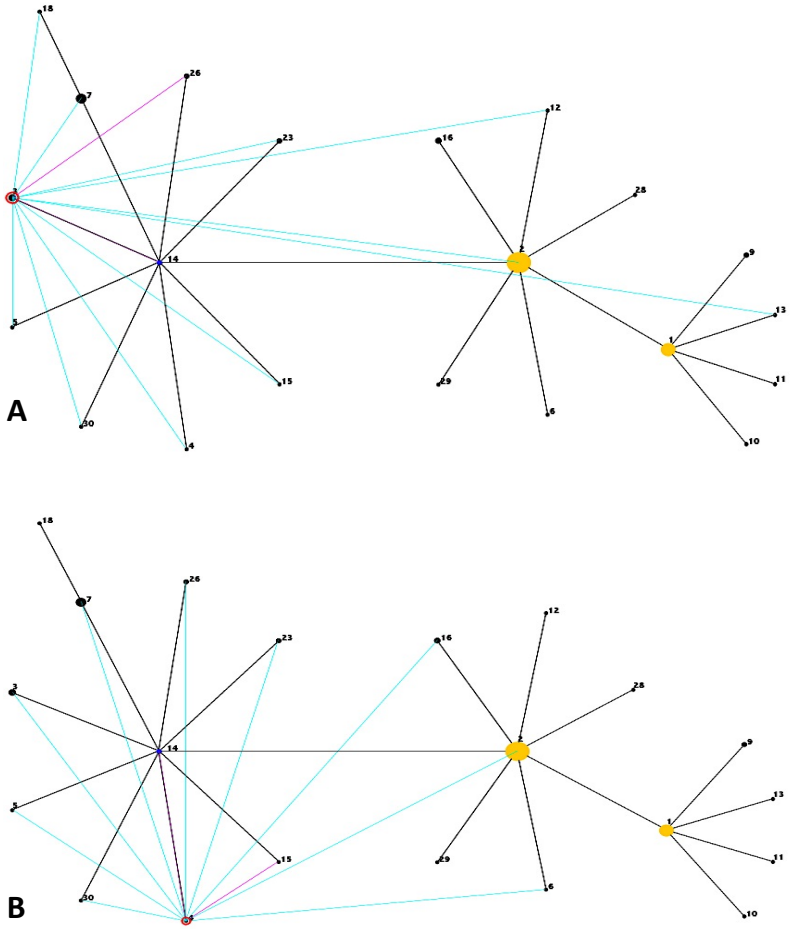


Figura complementaria 2. Complejo clonal 1 determinado por el análisis eBURST para *Yersinia ruckeri* en base al esquema de tipado de sequencias multilócicas (MLST). Los tipos de secuencias (ST) que se diferencian en un solo locus (SLV) se muestran conectados por líneas negras y/o rosa. Los STs que se diferencian en dos locus (DLV) se muestran conectados por líneas azules. El ST ancestral y los sub ancestrales se representan con un círculo azul y amarillo respectivamente. A) SLVs y DLVs para el ST 3. B) SLVs y DLVs para el ST 4.

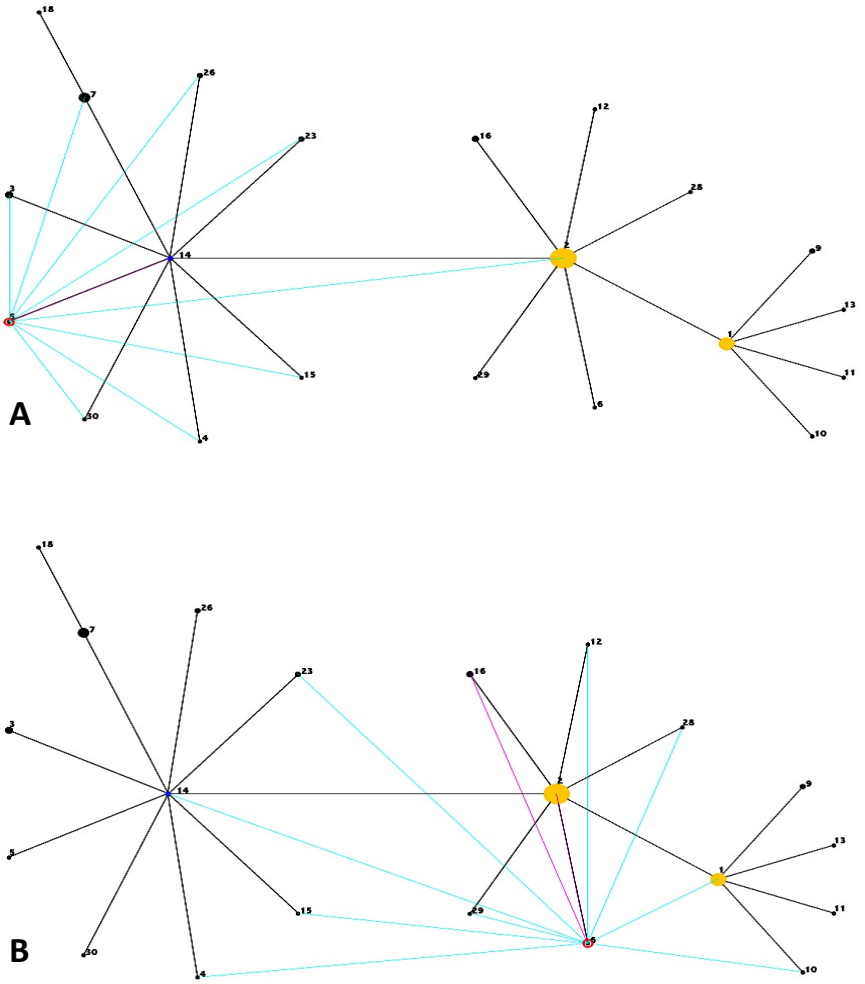


Figura complementaria 3. Complejo clonal 1 determinado por el análisis eBURST para *Yersinia ruckeri* en base al esquema de tipado de sequencias multilócicas (MLST). Los tipos de secuencias (ST) que se diferencian en un solo locus (SLV) se muestran conectados por líneas negras y/o rosa. Los STs que se diferencian en dos locus (DLV) se muestran conectados por líneas azules. El ST ancestral y los sub ancestrales se representan con un círculo azul y amarillo respectivamente. A) SLVs y DLVs para el ST 5. B) SLVs y DLVs para el ST 6.

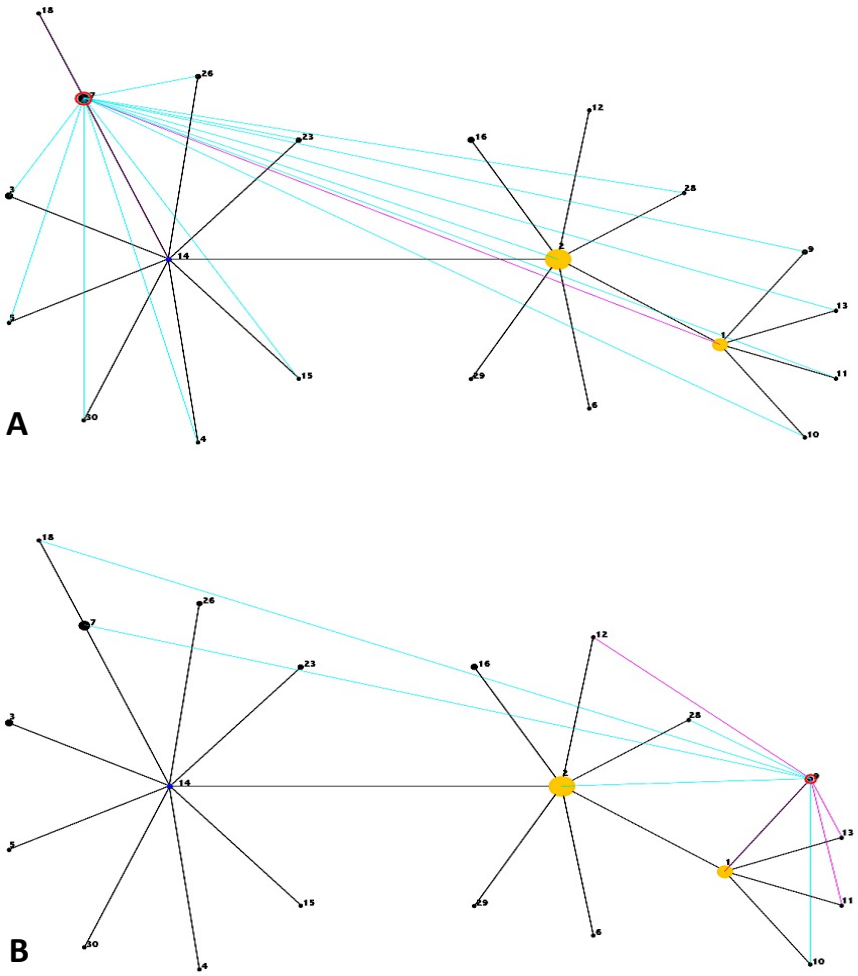


Figura complementaria 4. Complejo clonal 1 determinado por el análisis eBURST para *Yersinia ruckeri* en base al esquema de tipado de sequencias multilócicas (MLST). Los tipos de secuencias (ST) que se diferencian en un solo locus (SLV) se muestran conectados por líneas negras y/o rosa. Los STs que se diferencian en dos locus (DLV) se muestran conectados por líneas azules. El ST ancestral y los sub ancestrales se representan con un círculo azul y amarillo respectivamente. A) SLVs y DLVs para el ST 7. B) SLVs y DLVs para el ST 9.

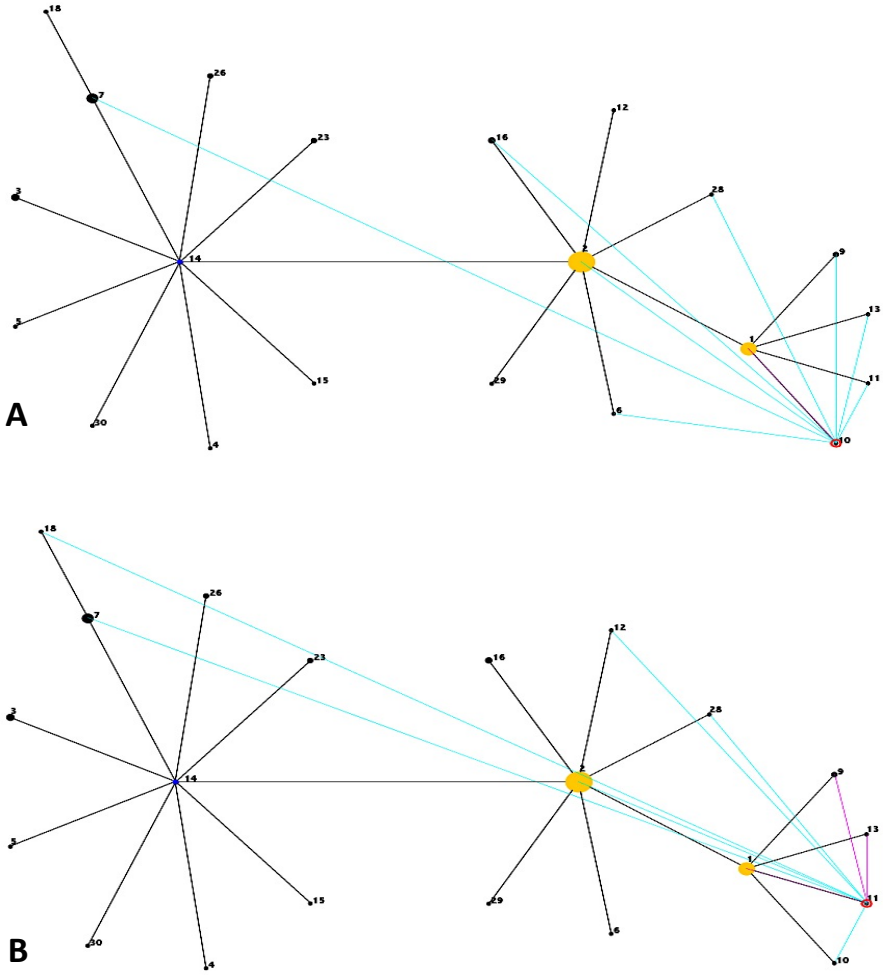


Figura complementaria 5. Complejo clonal 1 determinado por el análisis eBURST para *Yersinia ruckeri* en base al esquema de tipado de sequencias multilócicas (MLST). Los tipos de secuencias (ST) que se diferencian en un solo locus (SLV) se muestran conectados por líneas negras y/o rosa. Los STs que se diferencian en dos locus (DLV) se muestran conectados por líneas azules. El ST ancestral y los sub ancestrales se representan con un círculo azul y amarillo respectivamente. A) SLVs y DLVs para el ST 10. B) SLVs y DLVs para el ST 11.

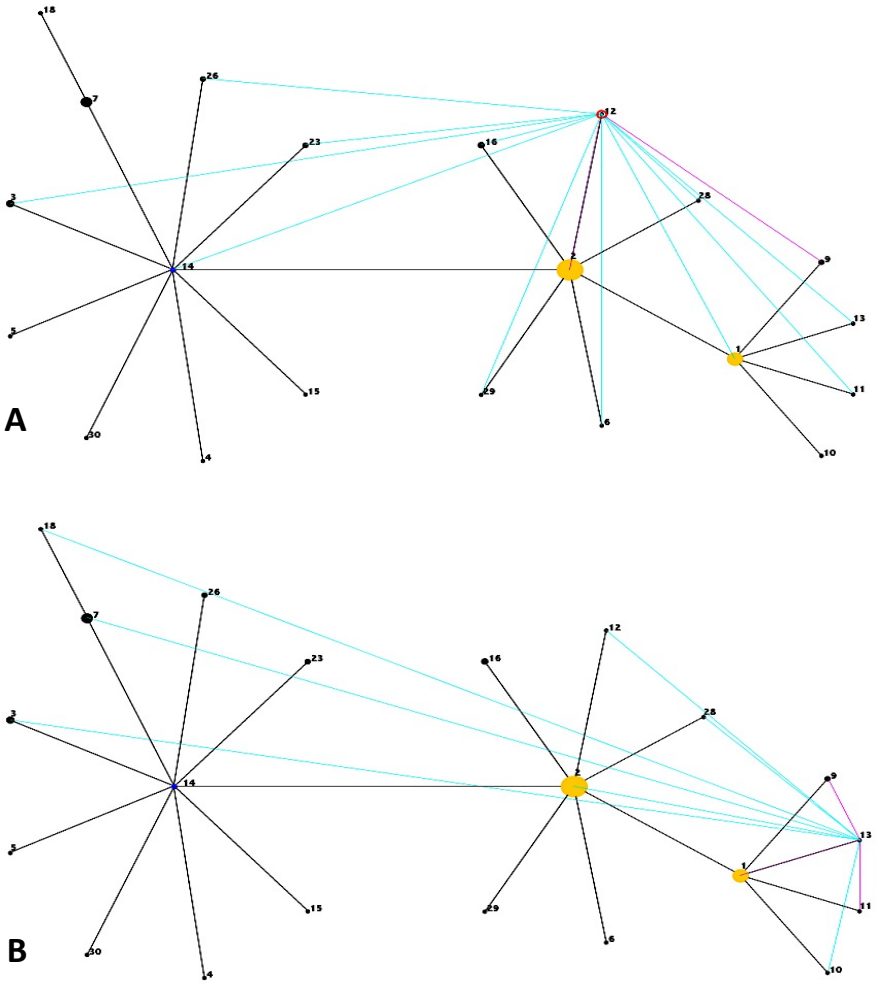


Figura complementaria 6. Complejo clonal 1 determinado por el análisis eBURST para *Yersinia ruckeri* en base al esquema de tipado de sequencias multilócicas (MLST). Los tipos de secuencias (ST) que se diferencian en un solo locus (SLV) se muestran conectados por líneas negras y/o rosa. Los STs que se diferencian en dos locus (DLV) se muestran conectados por líneas azules. El ST ancestral y los sub ancestrales se representan con un círculo azul y amarillo respectivamente. A) SLVs y DLVs para el ST 12. B) SLVs y DLVs para el ST 13.

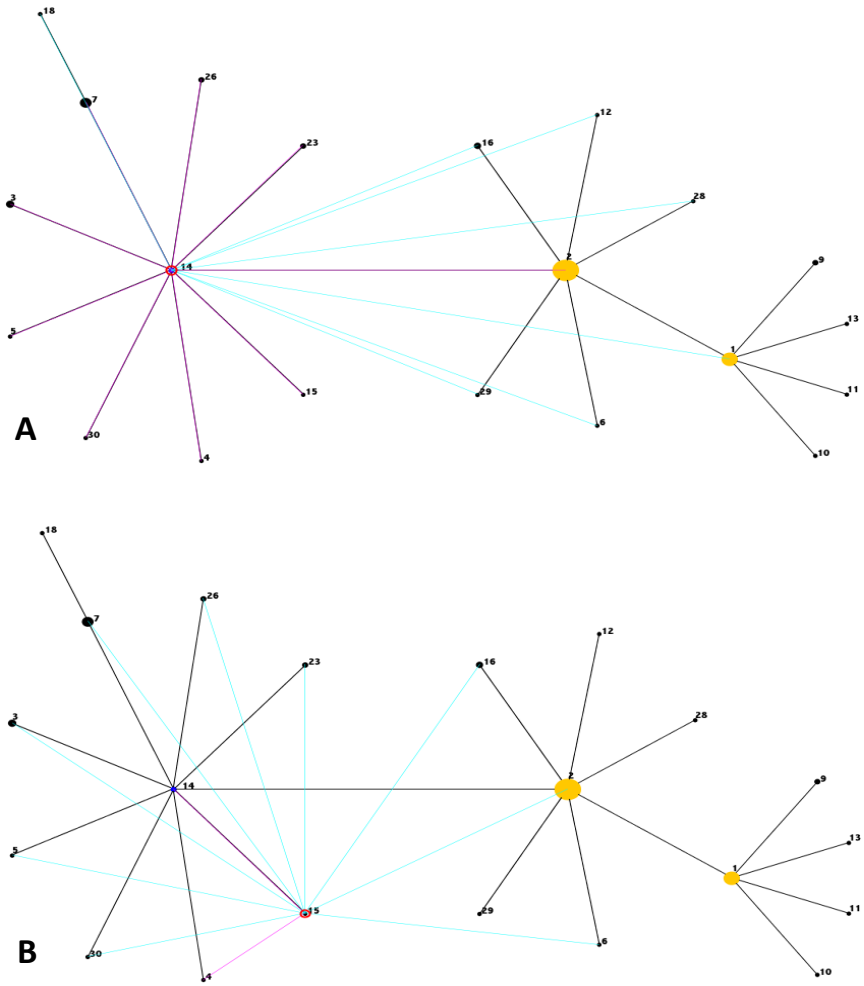


Figura complementaria 7. Complejo clonal 1 determinado por el análisis eBURST para *Yersinia ruckeri* en base al esquema de tipado de sequencias multilócicas (MLST). Los tipos de secuencias (ST) que se diferencian en un solo locus (SLV) se muestran conectados por líneas negras y/o rosa. Los STs que se diferencian en dos locus (DLV) se muestran conectados por líneas azules. El ST ancestral y los sub ancestrales se representan con un círculo azul y amarillo respectivamente. A) SLVs y DLVs para el ST 14. B) SLVs y DLVs para el ST 15.

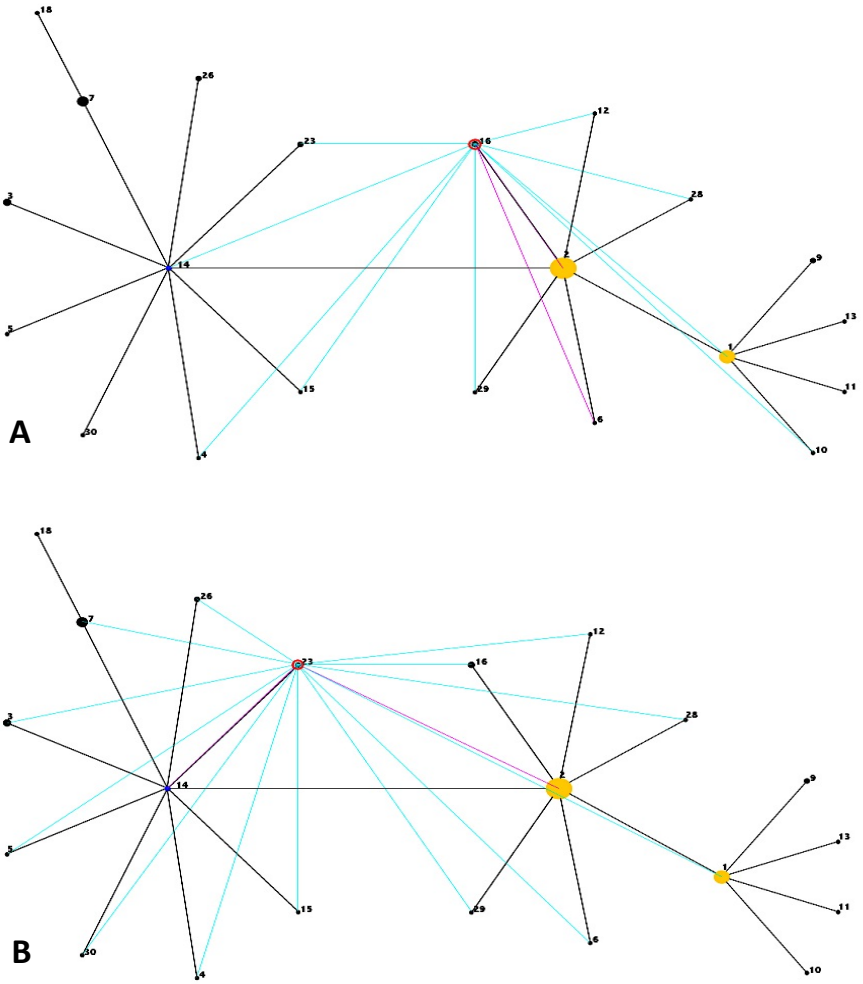


Figura complementaria 8. Complejo clonal 1 determinado por el análisis eBURST para *Yersinia ruckeri* en base al esquema de tipado de sequencias multilócicas (MLST). Los tipos de secuencias (ST) que se diferencian en un solo locus (SLV) se muestran conectados por líneas negras y/o rosa. Los STs que se diferencian en dos locus (DLV) se muestran conectados por líneas azules. El ST ancestral y los sub ancestrales se representan con un círculo azul y amarillo respectivamente. A) SLVs y DLVs para el ST 16. B) SLVs y DLVs para el ST 23.

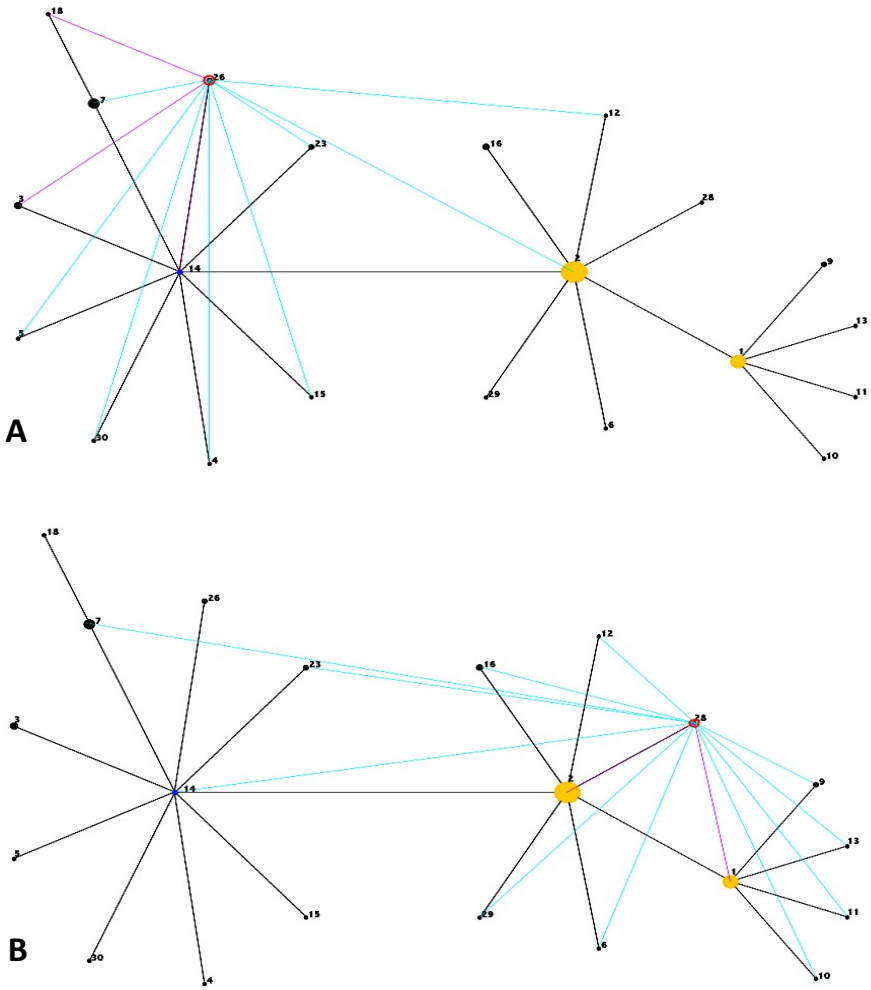


Figura complementaria 9. Complejo clonal 1 determinado por el análisis eBURST para *Yersinia ruckeri* en base al esquema de tipado de sequencias multilócicas (MLST). Los tipos de secuencias (ST) que se diferencian en un solo locus (SLV) se muestran conectados por líneas negras y/o rosa. Los STs que se diferencian en dos locus (DLV) se muestran conectados por líneas azules. El ST ancestral y los sub ancestrales se representan con un círculo azul y amarillo respectivamente. A) SLVs y DLVs para el ST 26. B) SLVs y DLVs para el ST 28.

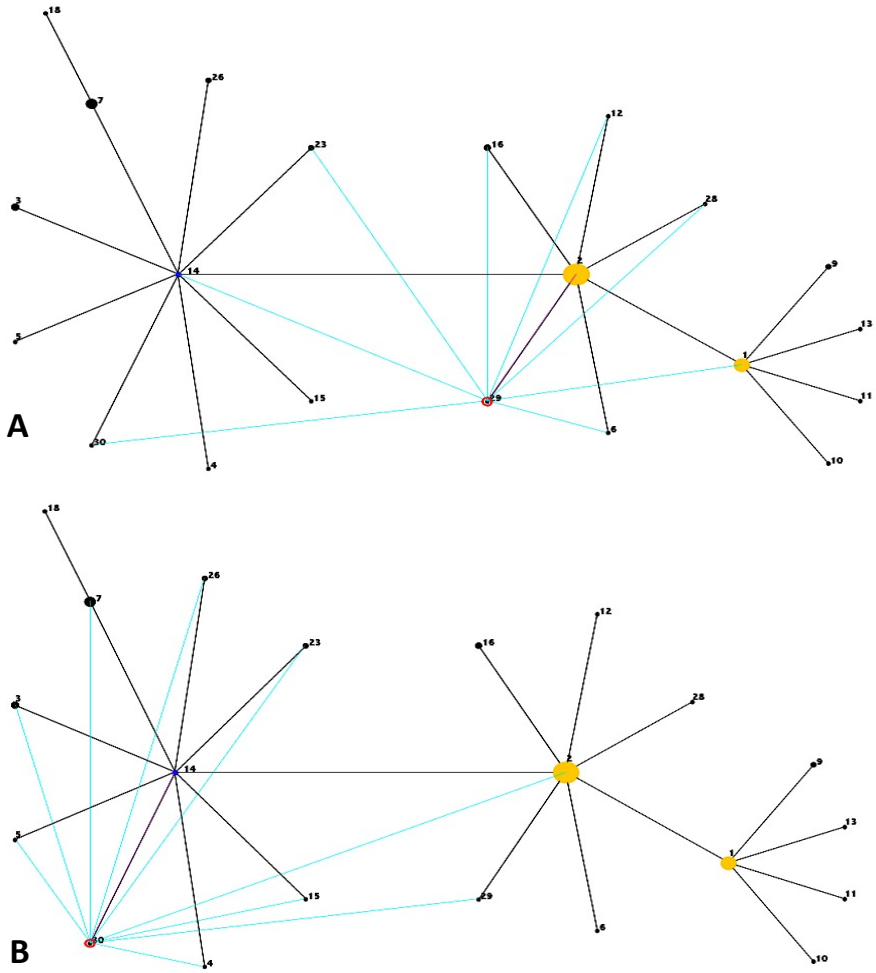


Figura complementaria 10. Complejo clonal 1 determinado por el análisis eBURST para *Yersinia ruckeri* en base al esquema de tipado de sequencias multilócicas (MLST). Los tipos de secuencias (ST) que se diferencian en un solo locus (SLV) se muestran conectados por líneas negras y/o rosa. Los STs que se diferencian en dos locus (DLV) se muestran conectados por líneas azules. El ST ancestral y los sub ancestrales se representan con un círculo azul y amarillo respectivamente. A) SLVs y DLVs para el ST 29. B) SLVs y DLVs para el ST 30.

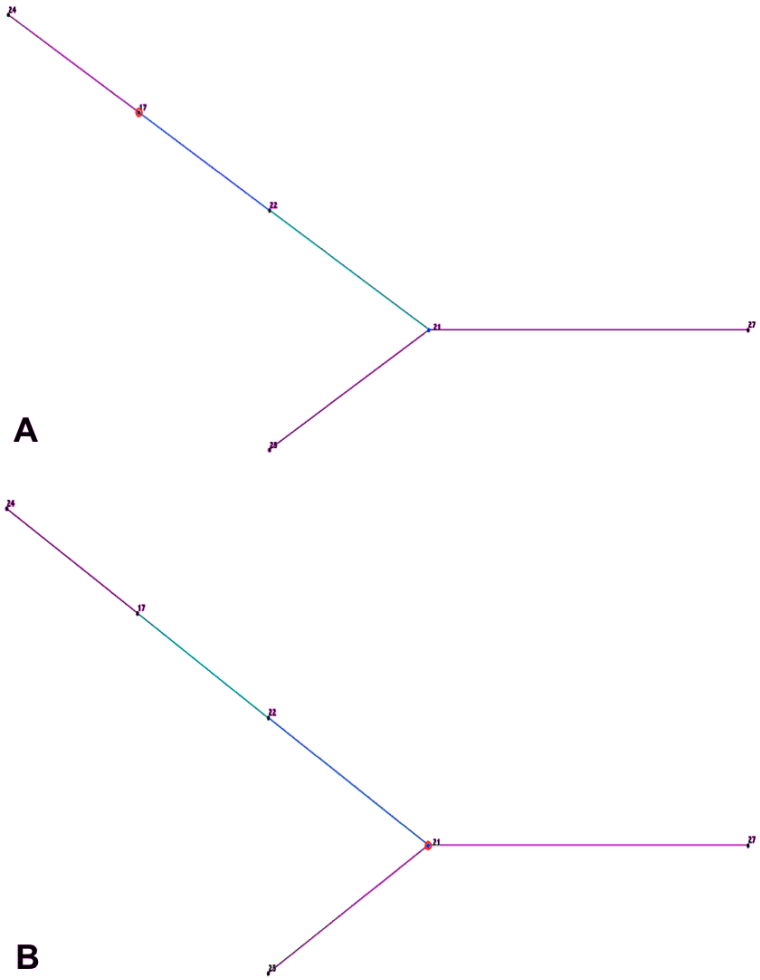


Figura complementaria 11. Complejo clonal 2 determinado por el análisis eBURST para *Yersinia ruckeri* en base al esquema de tipado de sequencias multilócicas (MLST). Los tipos de secuencias (ST) que se diferencian en un solo locus (SLV) se muestran conectados por líneas negras y/o rosa. Los STs que se diferencian en dos locus (DLV) se muestran conectados por líneas azules. El ST ancestral se representa con un círculo azul. A) SLVs y DLVs para el ST 17. B) SLVs y DLVs para el ST 21.

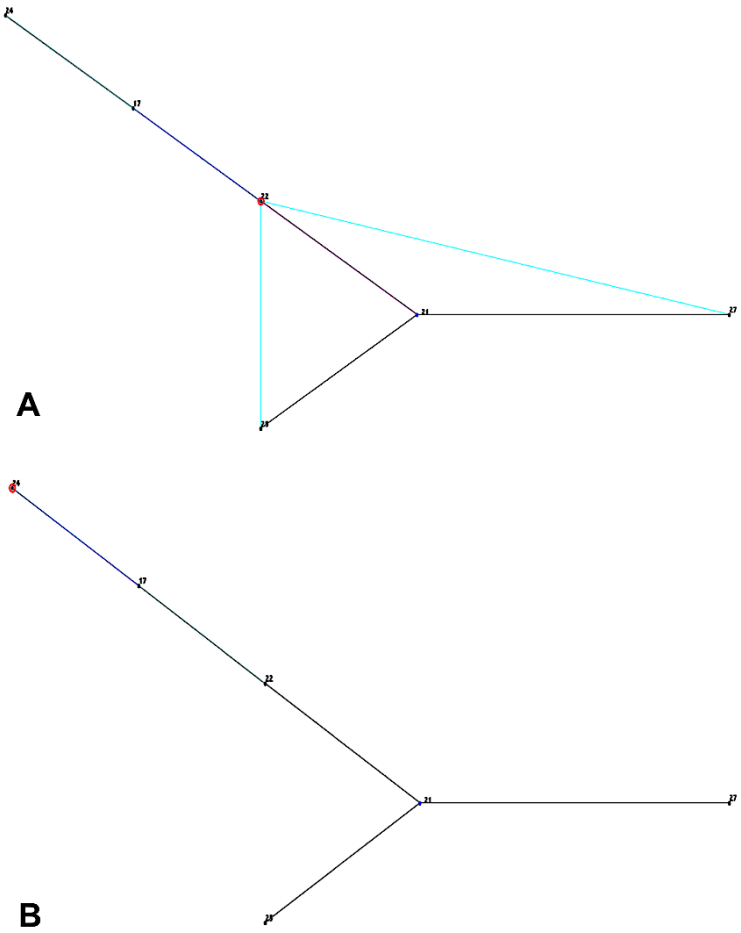


Figura complementaria 12. Complejo clonal 2 determinado por el análisis eBURST para *Yersinia ruckeri* en base al esquema de tipado de secuencias multilócicas (MLST). Los tipos de secuencias (ST) que se diferencian en un solo locus (SLV) se muestran conectados por líneas negras y/o rosa. Los STs que se diferencian en dos locus (DLV) se muestran conectados por líneas azules. El ST ancestral se representan se representa con un círculo azul. A) SLVs y DLVs para el ST 22. B) SLVs y DLVs para el ST 24.

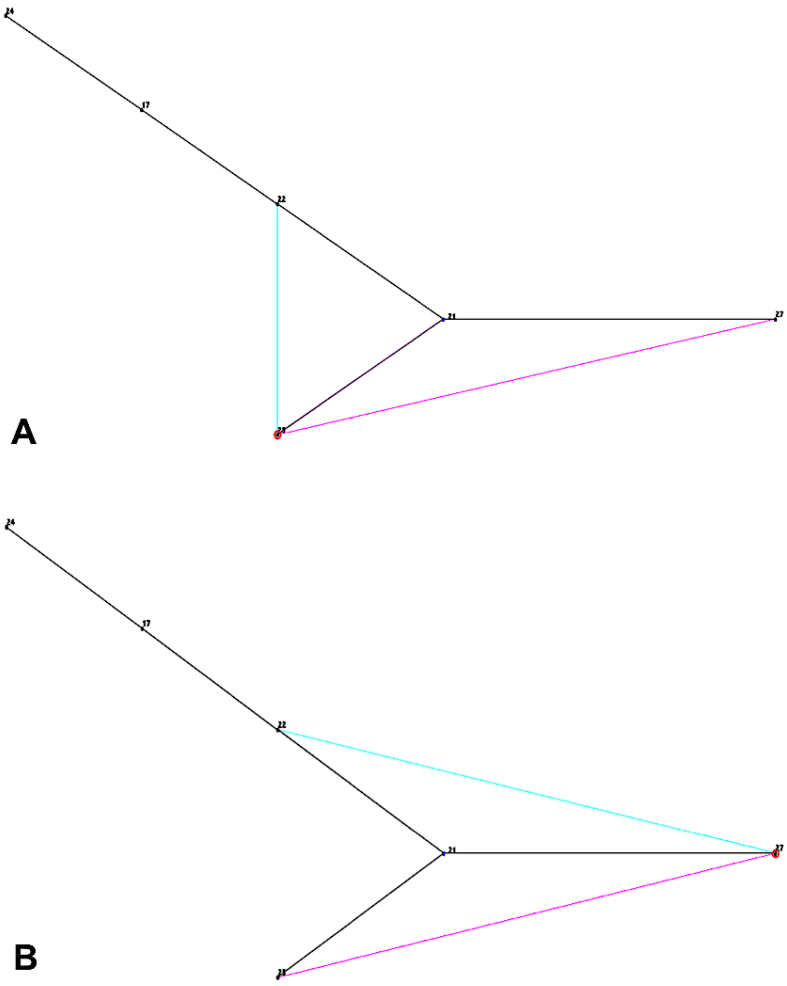


Figura complementaria 13. Complejo clonal 2 determinado por el análisis eBURST para *Yersinia ruckeri* en base al esquema de tipado de secuencias multilócicas (MLST). Los tipos de secuencias (ST) que se diferencian en un solo locus (SLV) se muestran conectados por líneas negras y/o rosa. Los STs que se diferencian en dos locus (DLV) se muestran conectados por líneas azules. El ST ancestral se representan con un círculo azul. A) SLVs y DLVs para el ST 25. B) SLVs y DLVs para el ST 27.

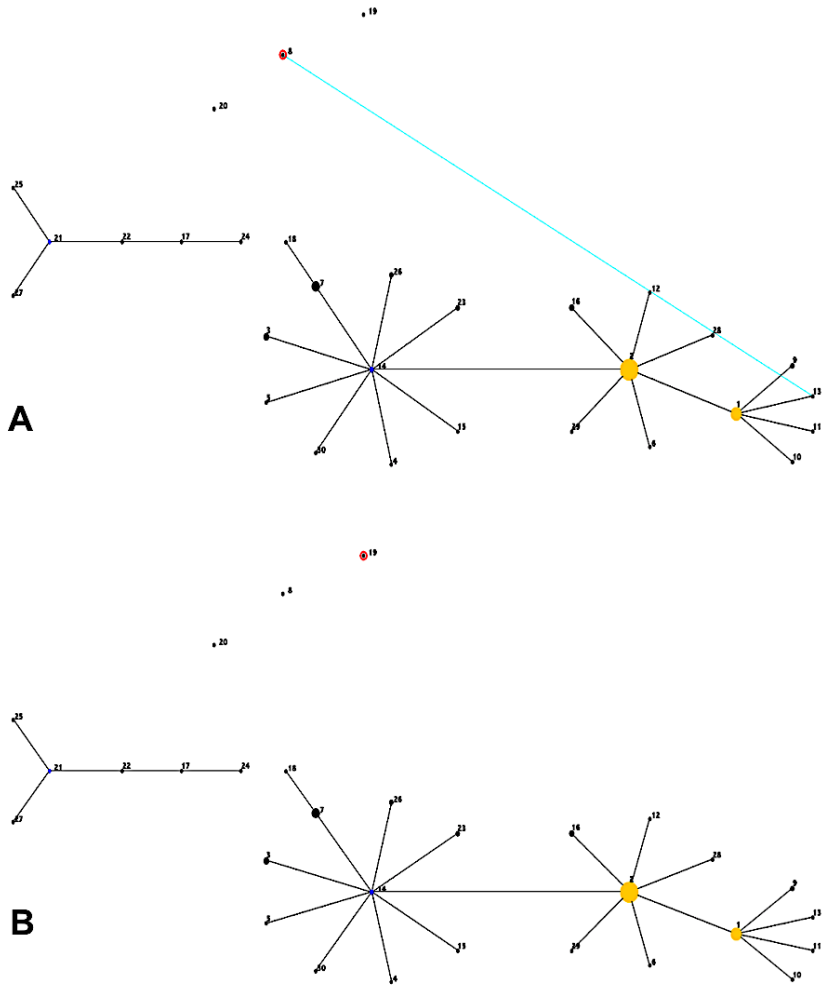


Figura complementaria 14. Estructura clonal determinada por el análisis eBURST para *Yersinia ruckeri* en base al esquema de tipado de secuencias multilócicas (MLST). Los tipos de secuencias (ST) que se diferencian en un solo locus (SLV) se muestran conectados por líneas negras y/o rosa. Los STs que se diferencian en dos locus (DLV) se muestran conectados por líneas azules. El ST ancestral y los sub ancestrales se representan con un círculo azul y amarillo respectivamente. A) SLVs y DLVs para la secuencia de tipo único ST 8. B) SLVs y DLVs para la secuencia de tipo único ST 19.

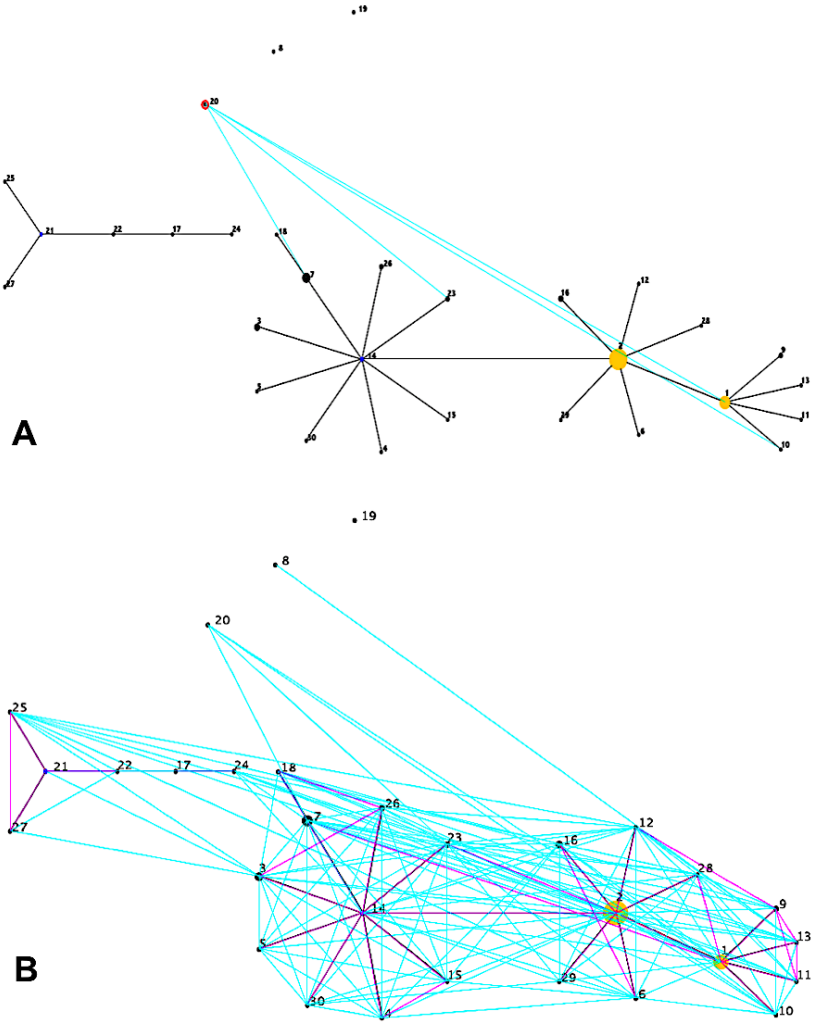


Figura complementaria 15. Estructura clonal determinada por el análisis eBURST para *Yersinia ruckeri* en base al esquema de tipado de secuencias multilócicas (MLST). Los tipos de secuencias (ST) que se diferencian en un solo locus (SLV) se muestran conectados por líneas negras y/o rosa. Los STs que se diferencian en dos locus (DLV) se muestran conectados por líneas azules. El ST ancestral y los sub ancestrales se representan con un círculo azul y amarillo respectivamente. A) SLVs y DLVs para la secuencia de tipo único ST 20. B) SLVs y DLVs para todos los STs establecidos.

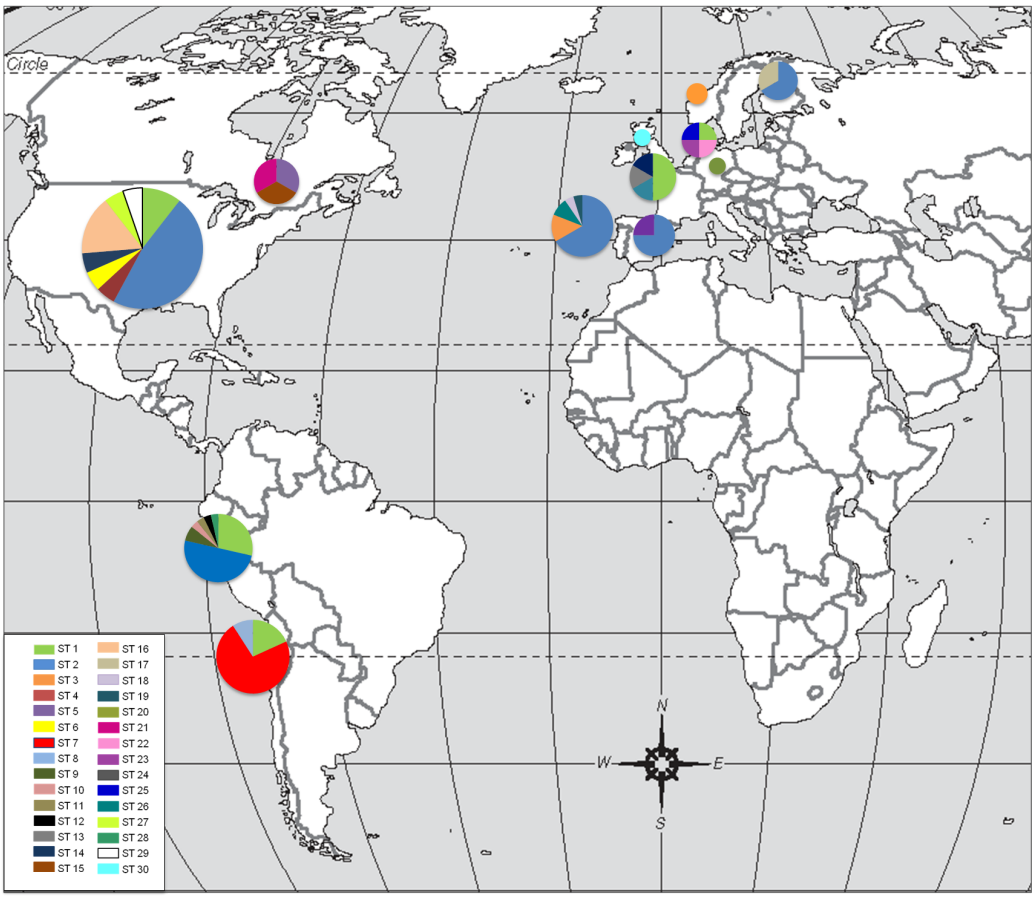


Figura complementaria 16. Distribución global de los diferentes tipos de secuencias (ST) establecidos para *Yersinia ruckeri* en base al análisis de tipado de secuencias multilócicas (MLST). (Tomado de: Bastardo, A., Balboa, S., Romalde J. L. (2012) From the gen sequence to the phylogeography through the population structure: the cases of *Yersinia ruckeri* and *Vibrio tapetis*. En *Protein and Nucleotide Sequence Analysis.* Publishing Services LLC, Cheyenne WY, USA)

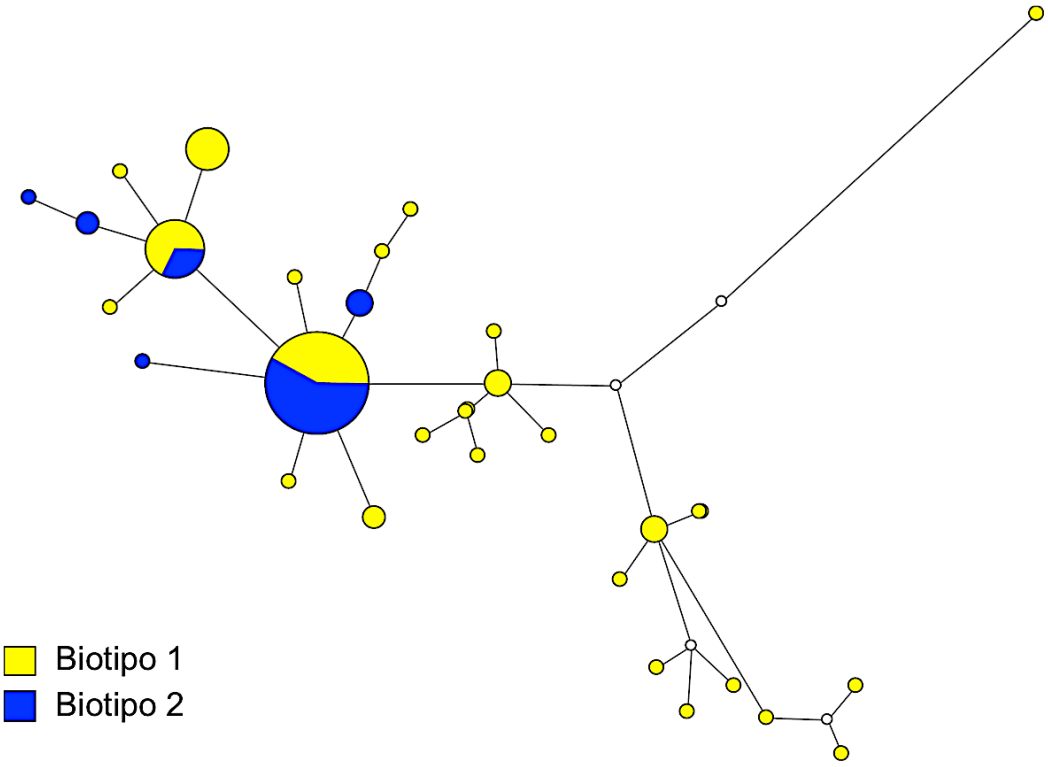


Figura complementaria 17. Red de haplotipos (95% de parsimonia) basada en los tipos de secuencias (ST) de *Yersinia ruckeri*. Cada circulo representa un ST. La línea entre los STs representan un cambio mutacional, los círculos abiertos representan STs no presentes en la población. El radio del los STs es proporcional al número de secuencias incluídos dentro de cada ST. El color de los círculos indica el biotipo de los aislados que conforman cada STs.

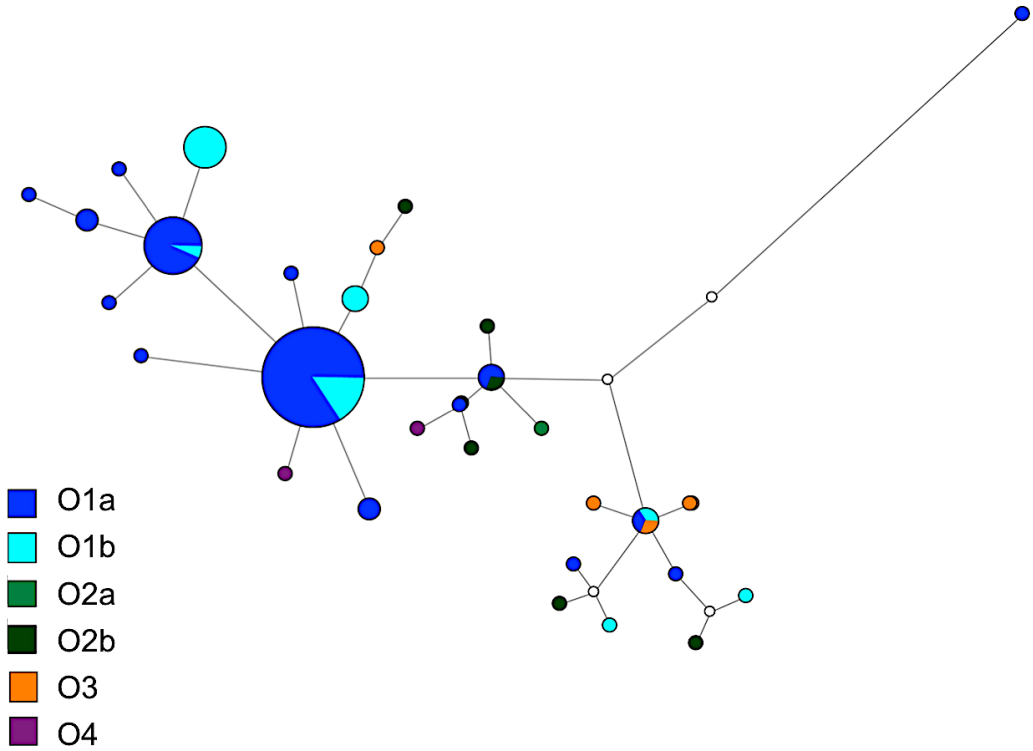


Figura complementaria 18. Red de haplotipos (95% de parsimonia) basada en los tipos de secuencias (ST) de *Yersinia ruckeri*. Cada circulo representa un ST. La línea entre los STs representan un cambio mutacional, los círculos abiertos representan STs no presentes en la población. El radio del los STs es proporcional al número de secuencias correspondiente a cada ST. El color de los círculos indican el serotipo de los aislados que conforman cada STs.

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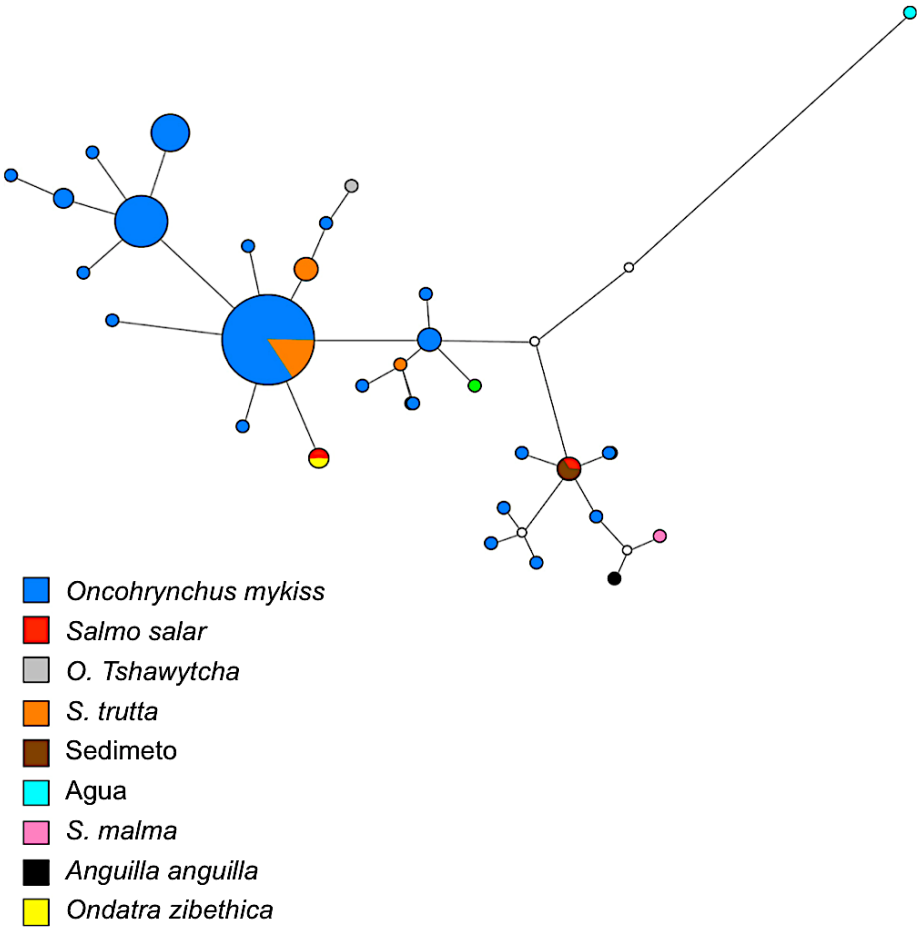

Figura complementaria 19. Red de haplotipos (95% de parsimonia) basada en los tipos de secuencias (ST) de *Yersinia ruckeri*. Cada circulo representa un ST. La línea entre los STs representan un cambio mutacional, los círculos abiertos representan STs no presentes en la población. El radio del los STs es proporcional al número de secuencias correspondiente a cada ST. El color de los círculos indican la fuente de aislamiento (huésped o ambiental) de los aislados que conforman cada STs.

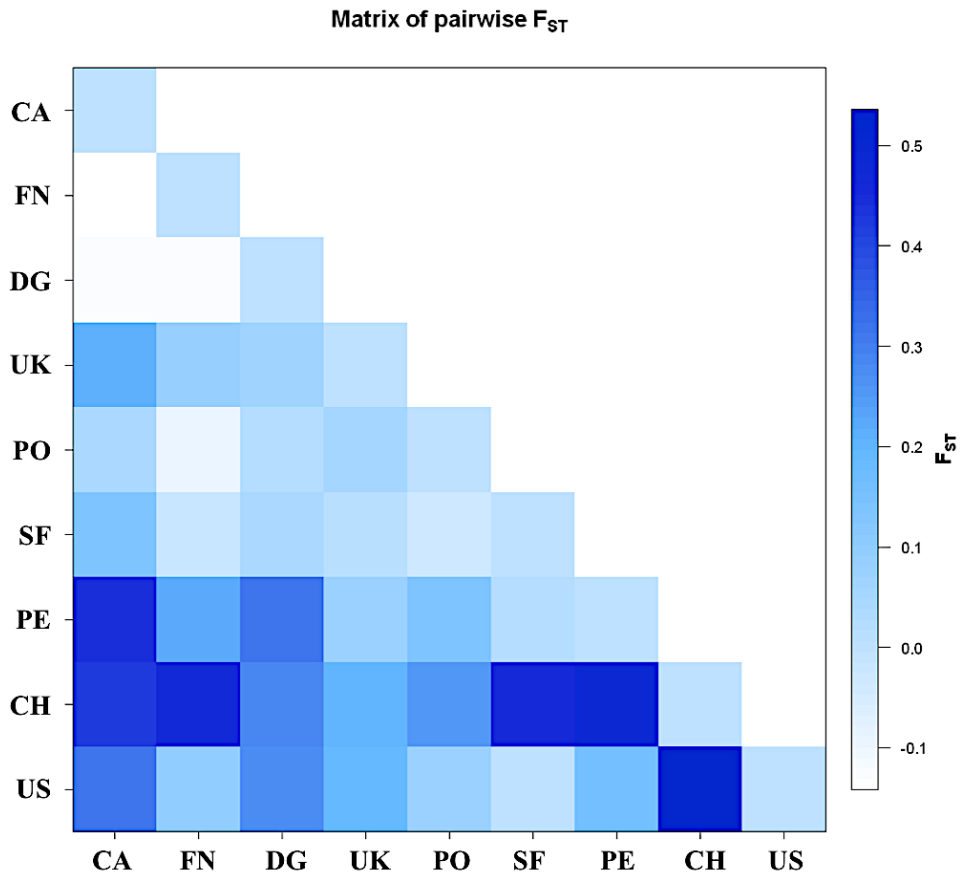

Figura complementaria 20. Matriz de diferenciación genética (F_{ST}) entre pares de subpoblaciones de *Yersinia ruckeri.* CA, Canadá; FN, Finlandia-Noruega; DG, Dinamarca-Alemania; UK, Reino Unido; PO, Portugal; SF, España-Francia; PE, Perú; CH, Chile, US, USA.

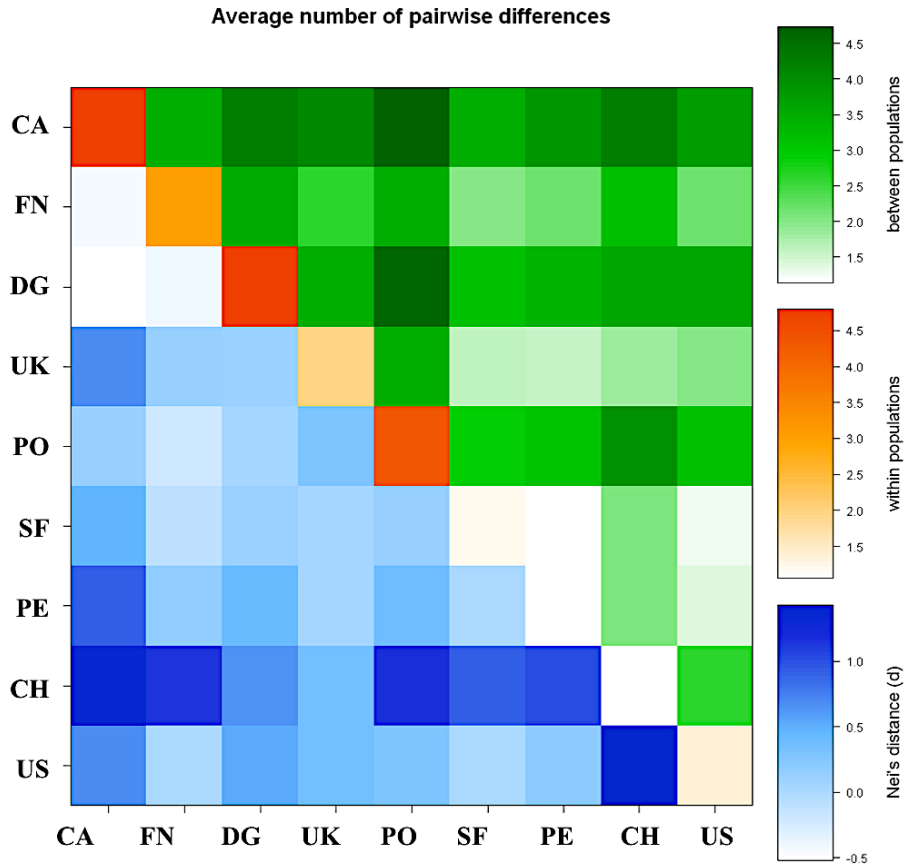

Figura complementaria 21. Matriz del número promedio de diferencias entre y dentro de las subpoblaciones de *Yersinia ruckeri.* CA, Canadá; FN, Finlandia-Noruega; DG, Dinamarca-Alemania; UK, Reino Unido; PO, Portugal; SF, España-Francia; PE, Perú; CH, Chile, US, USA.

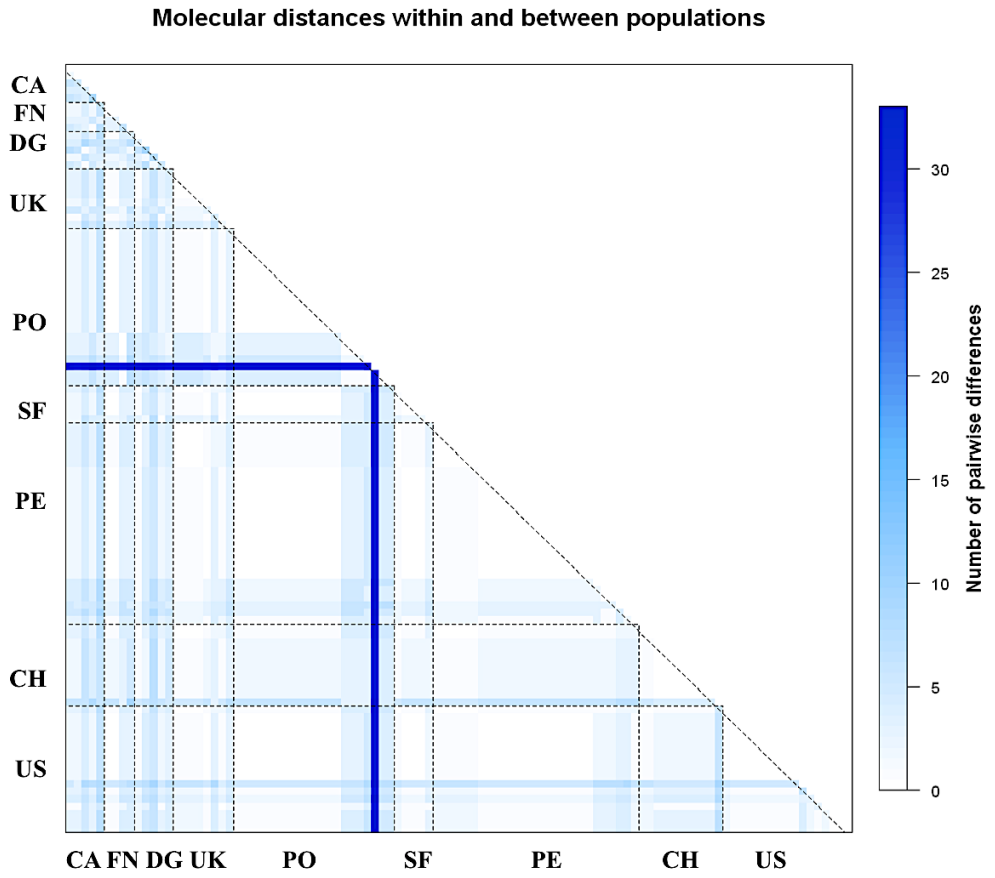

Figura complementaria 22. Matriz de diferenciación molecular dentro y entre pares de subpoblaciones de *Yersinia ruckeri*. CA, Canadá; FN, Finlandia-Noruega; DG, Dinamarca-Alemania; UK, Reino Unido; PO, Portugal; SF, España-Francia; PE, Perú; CH, Chile, US, USA.

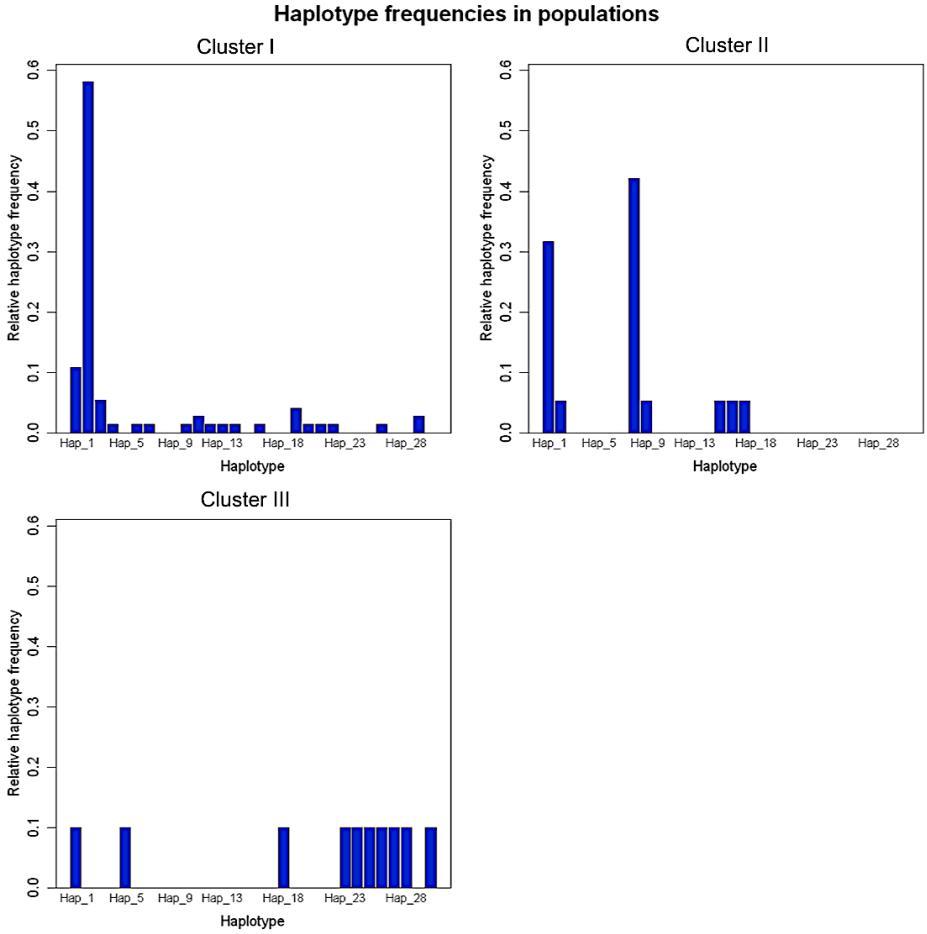

Figura complementaria 23. Frecuencia de haplotipos en cada uno de los grupos genéticos (Cluster I, II y III) determinados para la población de *Yersinia ruckeri* mediante el análisis STRUCTURE.

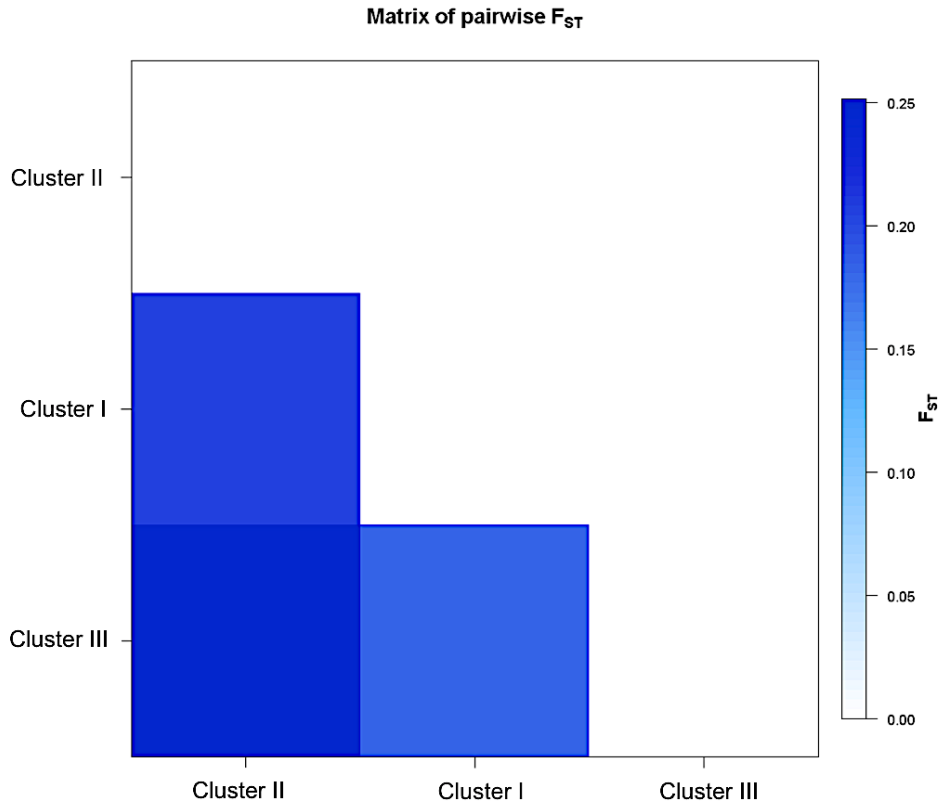

Figura complementaria 24. Matriz de diferenciación genética (F_{ST}) entre pares de grupos genéticos (Cluster I, II y III) determinados para la población de *Yersinia ruckeri* mediante el análisis STRUCTURE.

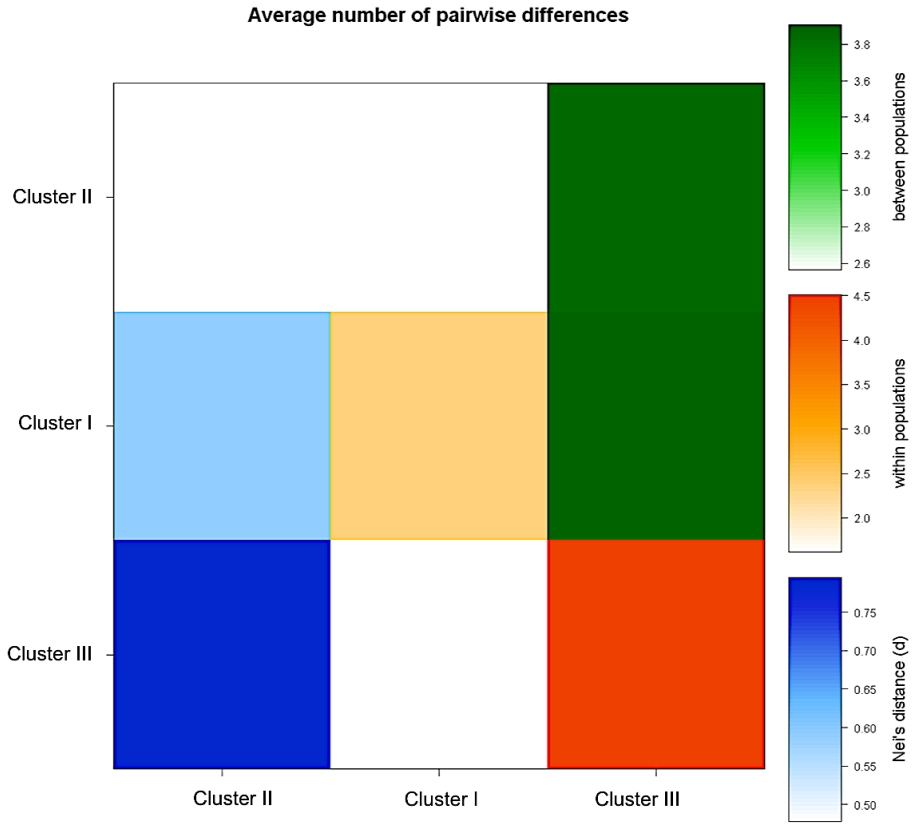

Figura complementaria 25. Matriz del número promedio de diferencias dentro y entre los grupos genéticos (Cluster I, II y III) determinados para la población de *Yersinia ruckeri* mediante el análisis STRUCTURE.

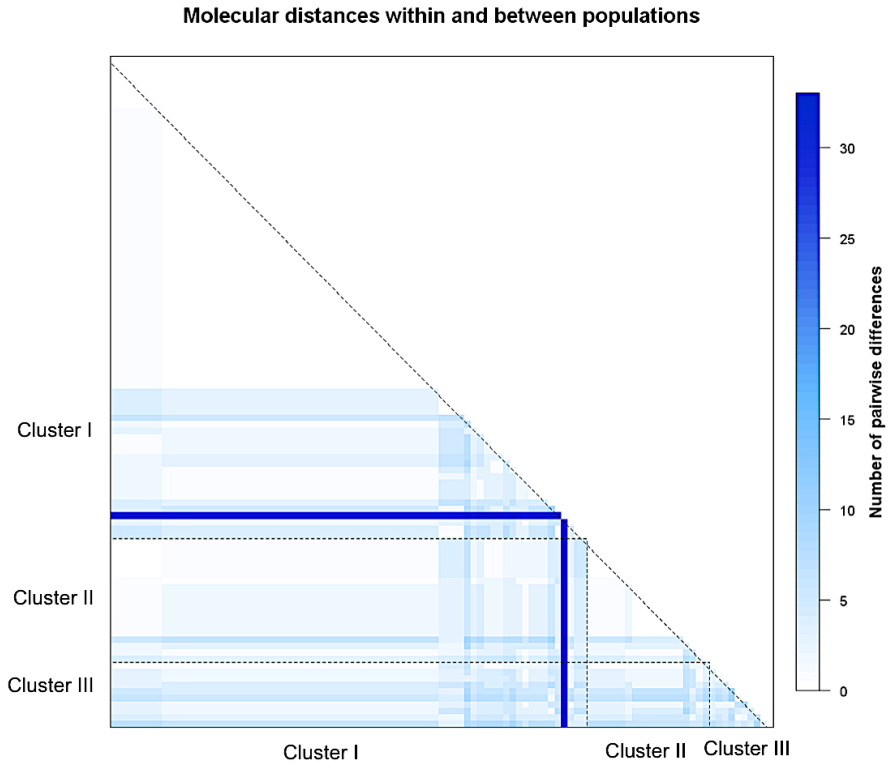

Figura complementaria 26. Matriz de diferenciación molecular dentro y entre pares de los grupos genéticos (Cluster I, II y III) determinados para la población de *Yersinia ruckeri* mediante el análisis STRUCTURE.

5. CAPÍTULO III:

EVOLUCIÓN MOLECULAR DE *Yersinia ruckeri*

Artículo:

Aquaculture drives fish pathogen evolution: the *Yersinia ruckeri* tale.

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Referencia:

Manuscrito en preparación.

Resumen:

El patógeno bacteriano de peces *Yersinia ruckeri* se ha controlado con éxito mediante la vacunación durante décadas, pero recientemente han vuelto a surgir brotes de yersiniosis en el cultivo de salmónidos. En este trabajo, se presentan evidencias de que esta especie ha evolucionado en respuesta a las operaciones de acuicultura, como la transferencia de peces entre países y la vacunación. Sobre la base del análisis de secuencias de genes esenciales se ha reconstruido la historia de la divergencia y la propagación de este patógeno. El ancestro común más reciente se ha datado aproximadamente cinco siglos atrás, y los resultados mostraron que USA tiene la mayor probabilidad de ser el origen de la difusión desde 1950. La alta tasa de sustitución observada podría explicar el hecho de que las cepas emergentes resistentes a la vacuna han derivado de un ancestro datado entre veinte y seis años atrás. Además, este trabajo constituye un modelo útil para el estudio de la influencia de factores antropogénicos sobre la evolución de otros patógenos animales y humanos.

AQUACULTURE DRIVES FISH PATHOGEN EVOLUTION: THE *Yersinia ruckeri* TALE.

Asmine Bastardo[1, 2], Carmen Ravelo[2], and Jesús L. Romalde[1]

SUMMARY

The bacterial fish pathogen *Yersinia ruckeri* has been successfully controlled by vaccination during decades, but yersiniosis outbreaks have recently re-emerged in the salmonid industry. Here we provide evidence that this species has evolved in response to aquaculture operations such as worldwide fish transfer and vaccination. On the basis of the sequence analysis of housekeeping genes we have reconstructed the historical divergence and spread of this pathogen. The most recent common ancestor existed approximately five centuries ago, and the findings showed that USA had the highest probability of being the origin of the spread since 1950s. The high substitution rate determined might explain the fact that the emergent vaccine resistant strains have just derived from an ancestor living twenty to six years ago. In addition, this work constitutes a useful model for the study of the influence of anthropogenic factors on the evolution of other animal and human pathogens.

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Phylogeographic analyses are a common approach in molecular ecology, connecting historical processes in evolution with spatial distribution that traditionally scale over millions of year[1]. Due to the lack of fossil evidence, the timescale of bacterial evolution are largely unknown. For bacterial pathogens, these spatio-temporal reconstructions may provide insights into the origin and epidemic spread beyond the predictions arising from standard epidemiological surveillance. This has motivated the development of analytical tools to uncover the footprint of spatial history in contemporaneous molecular sequences[2-4].

The Gram-negative bacterium *Y. ruckeri* is historically one of the most important recognized fish pathogens and the etiological agent of yersiniosis or enteric redmouth (ERM) disease, a systemic infection of salmonid fish that causes important economic losses worldwide. This pathogen was first isolated in the 1950s from rainbow trout *Oncorhynchus mykiss* (Walbaum), in the Hagerman Valley of Idaho, USA. Since the *Y. ruckeri* description, the biotype 1 (positive for lipase and motility tests) strains, belonging to serotype O1a (Hagerman strain), have remained endemic and widely found in all salmonid-producing countries.

Although ERM has been successfully controlled for decades by vaccination, recent outbreaks have occurred in vaccinated fish at farms in the southern USA and throughout Europe, mainly associated to an emerging biotype 2 (negative for lipase and motility tests) within the serotype O1a[5-8]. Other epizootics have been also reported being caused by uncommon serotypes in vaccinated rainbow trout in Spain[9] and in vaccinated Atlantic salmon (*Salmo salar*) in Australian and Chilean hatcheries[10,11].

Although it was believed that this species represented a single clone widely distributed, multilocus sequence typing (MLST)[12] has recently provided new evidences on the population structure of *Y. ruckeri* and on the genetic mechanisms

behind the emergence of clonal lineages, showing an epidemic model of clonal expansion for *Y. ruckeri*. However, the epidemiological history of this pathogen is still unclear because of the lack of appropriate studies.

Here we employed a Bayesian approach to reconstruct the transmission and divergence of the fish pathogen *Y. ruckeri* through time and space, and to infer the evolutionary processes of this bacterium. Our phylogenomic analyses provide important insights into the evolution and diversification of *Y. ruckeri*.

Rates of nucleotide substitution in *Y. ruckeri*

A measure of the nucleotide substitution rate in natural populations of pathogenic bacteria enable the dating of evolutionary events and the reconstruction of their demographic history, providing also fundamental insights into the forces driving disease emergence and spread[13,14]. However, the speed at which nucleotide substitutions occurred in bacterial populations has rarely been determined[15-17]. We employed a Bayesian Monte-Carlo Markov chain (MCMC) approach[18] (see Methods) to determine the mean nucleotide substitution rate in six housekeeping (HK) genes of *Y. ruckeri,* and for the concatenation of these sequences, which resulted in a final fragment of 2,876 bp (Table 1). In all data sets analyzed, we obtained high values of the coefficient of variation (CoV) under the relaxed molecular clock (mean values > 2) supporting non-clock-like behaviour[19] (i.e., rate variation among lineages) and large-scale rate variation among *Y. ruckeri* lineages. Our findings point to a rapid clonal evolution of *Y. ruckeri.* The substitution rate was similar among the *Y. ruckeri* HK genes, with values estimated to be between 1.1×10^{-5} and 2.6×10^{-5} substitutions per site per year. Furthermore, for the concatenated sequences, the nucleotide substitution rate was

estimated to be 2.5 x 10^{-5} substitutions per site per year (95% HPD= 1.4 x 10^{-5} to 4.2 x 10^{-5}). This value exceeds the evolutionary rate estimation proposed for *Escherichia coli* (3 x 10^{-8} substitutions per nucleotide site and year) by almost three orders of magnitude[20]. However, this estimation had been based on a laboratory mutation rate of 10^{-10} per nucleotide site and generation, and on the assumption of approximately 300 generations elapsing per year, which have been demonstrated to vary widely among bacteria species[21,22]. Disentangling the effects of recombination and point substitution can be challenging and some previously published substitution rates are likely to be upper bounds rather than point estimates[23]. Higher upper limits of substitution rates in bacteria have been also estimated for *Helicobacter pylori* (4.1 x 10^{-5}), *Yersinia pestis* (7.42 x 10^{-7})[24], and *Staphylococcus aureus* (2 x 10^{-6})[23]. Some degree of time dependence has been observed in rate estimations from bacteria, although there is a evident lack of studies on bacterial rates across significant evolutionary timescales[25].

Time of the more recent common ancestors (TMRCA)

Genetic sequences provide a unique source of information about the epidemiological connections between bacteria sampled through time and across space[26]. Thus, bacterial genetics can be useful to illuminate the origins of an epidemic. To understand the diversification of *Y. ruckeri* over time, we also used the Bayesian approach to determine the dates of the more recent common ancestors (MRCA) that have originated the different currently recognized O-serotypes[27] of *Y. ruckeri*, as well as the divergence time of the isolates associated to recent ERM outbreaks in vaccinated fish (Supplementary Table 1). These dates were obtained from Bayesian time stamped maximum clade credibility (MCC)

tree constructed separately for partial sequences of each HK gene (Supplementary Figs. 1-3), and for the concatenated sequences (Supplementary Fig. 4). All serotypes have diverged during the same period, i.e. before 1970. The oldest serotypes identified were the O2a and O1a with MRCA existing in 1917 (95% HPD: 1842-2004) and 1937 (95% HPD: 1889-1960), respectively. The youngest serotype of *Y. ruckeri* was serotype O1b, with a MRCA from 1979 (95% HPD: 1914-1985). Although some O-serotype representative strains seem to share the same MRCA in the MCC tree of different HK genes, the MCC tree of the concatenated sequence indicates that they derived, in most of cases, from different MRCA (Supplementary Fig. 4). Thus, previously described North American serotypes[27] are distinct in the phylogeny, and the temporal reconstruction discovered different ancestors. Divergence of other strains of *Y. ruckeri* belonging to the different serotypes over time was also observed to occur from different ancestors and in different geographical areas (Supplementary Fig. 4). This study revealed that multiple *Y. ruckeri* lineages have emerged independently from different ancestors over time.

The TMRCA for biotype 2 of *Y. ruckeri* was determined to be 1980 (95% HPD= 1962-1987) based on the emergence of the oldest non-motile strain (isolated in 1995 from UK) included in the concatenated sequences analysis (Table 2 and Supplementary Fig. 4). This ancestor was also shared with other motile strains isolated from Europe. Different lineages were recognized for the USA, Portugal, Peru, Finland, and UK between 2001 and 2009 for the remaining biotype 2 *Y. ruckeri* virulent strains (Table 2). Furthermore, the virulent motile *Y. ruckeri* strains, belonging to the serotype O1b and associated to important outbreaks in vaccinated salmon occurred in 2008 in Chile, were found to be originated from an ancestor existing in 1983 (95% HPD, 1971-1987). While, the motile serotype O2b

Y. ruckeri reported first in Spain in 2002, and associated with mortalities of vaccinated rainbow trout, was dated to have emerged from an ancestor of 1970 (95% HPD= 1940-1983). These results confirmed that new virulent *Y. ruckeri* lineages are currently circulating in North America, Europe and South America. The extent of *Y. ruckeri* subdivision between countries indicates that for six of thirteen countries sampled (i.e. USA, Peru, Portugal, UK, Chile and Canada) the observed genetic divergence could be derived by *in situ* evolution after extensive migration.

Analysis of spatio-temporal spread dynamics

Reconstructing the evolutionary history and spatial processes from genetic sequences in microorganisms can provide fundamental understanding of the evolutionary dynamic underlying epidemics[28,29]. These insights could be properly used to implement an effective intervention and preventive strategies. Here we quantified the rate at which the first *Y. ruckeri* linages have moved among sampled geographical locations over time by a discretized spatial diffusion model[26] (see Methods). The most probable location for each node was annotated via color-labeling of the branches on the MCC tree (Fig. 1). Our findings provide evidence of gene flow between countries facilitated by the early movement of carrier fish during the expansion of salmonid aquaculture around the world. USA was identified as the predominant location of the oldest sequences throughout the evolutionary history of the *Y. ruckeri*. The posterior distribution values indicated highest probability (PP) for USA (64%) as the locality origin of the spread processes (MCC tree root in Fig. 1), and for the MRCA origin (see histogram inserted in Fig. 1). While the earliest divergence likely occurred in Canada in

1972 (95% highest posterior density values (HPD)= 1964-1978) with origin in USA (PP= 92%), the epidemic was independently seeded on many European locations starting from around 1979 (95% HPD= 1972-1980) in France, which resulted in a high diversity of European lineages in 1990. With some exceptions of direct Canada-Europe transmissions, most European lineages appear to be originating from the USA. Movement from USA to South America was detected by the location of the 2008 Peruvian isolates that had 85% probability of being originated from USA. There is 54% probability that Peruvian isolates were also transmitted from UK. Similarly, transference of *Y. ruckeri* from UK to Chile was also supported (60% probability).

The emergence of the virulent biotype 2 strains in UK occurred approximately 15 years after the introduction of *Y. ruckeri* into Europe (1994, 95% HPD= 1988-1995), being originated in the UK itself from a motile isolate (PP=62%). Although we evidenced other ancestral node for the subsequent appearances of non-motile UK isolates in 2001, these were also located deriving from UK (PP= 69–91%) indicating independent emergence over time. The biotype 2 strains associated to the epizooties occurred between 2003 and 2005 in USA also emerged from a motile ancestor located in USA (PP= 76%). There was 53% probability that the common ancestor of the 2006 Portuguese non-motile linage was also located in USA. Furthermore, independent introductions of non-motile strains from USA and UK into Peru, were also statistically supported (PP= 85% and PP= 60%, respectively).

The MCC tree provided a glimpse into *Y. ruckeri* spatial diffusion over time. However, the showed scenario is derived from a single tree in the posterior distribution. To better depict the global spread of the epidemic over time, we reconstructed movements displayed sequentially over time (Fig. 2 and

supplementary video). In addition, to establish epidemiological linkage while taking into account phylogenetic uncertainty, we employed a Bayesian stochastic search variable selection (BSSVS) procedure using a prior distribution that employ a minimal set of location exchange rates to explain the diffusion patterns[26] (see Methods).

This analysis resulted in a posterior mean of 14 (13-17) non-zero rates which revealed globally epidemiological linkages (Fig. 3 and Supplementary Table 2). Most of the transmission pathways inferred from USA were statically supported, and the majority of these epidemiological pathways represent long-distance linkages, which is consistent with long-distance spread of the *Y. ruckeri* through fish movements during aquaculture expansion. The strongest link is observed between Denmark and Chile (Bayes factor= 25). Although high probability of ancestral node in Denmark for Chile was not previously evidenced, they could be indirectly connected through UK-Denmark link (Fig. 3). USA was involved in five links with a Bayes factor > 5 that included the earliest dispersal event between USA and Canada as evidenced by the MCC tree (Fig. 1). Conversely, the earlier dispersal event between USA and Europe (France) does not yield statistical support. Whereas European locations like Norway, Scotland and Spain are directly linked to USA, others like Portugal, Denmark, Germany and France could only be indirectly connected to USA (via either Scotland or Norway). Europe has multiple links with USA and Canada, but only Norway, Portugal and UK also established epidemiological linkage with South America (Chile and/or Peru). Within Europe, both short and long distance connections make up a complex network of epidemiological linkage.

Demographic expansion

Genomes of modern representative populations contain signatures about the demographic history from which they originate[29]. Thus, estimations of effective population size and the rate of change through time, provide valuable information on the evolutionary and demographic processes of a population. We have showed that *Y. ruckeri* has accumulated measurable genetic changes within an epidemiological timescale. A Bayesian skyline plot indicated a very initial stable population with a sharp increase of the effective population size starting in the 1950s, which continues increasing for twenty-five years (Fig. 4). This demographic expansion, including the timing of events, is in full agreement with the observation of *Y. ruckeri* emergence in USA and Europe. This also corresponded with the period when each of the major lineages diverged (Fig.1) and subsequently became widespread in salmonid farming throughout of world[12]. The combination of ecological and evolutionary factors led to this rapid increase in diversity, namely, the spread of the ERM disease through large, immunologically naive salmonid populations, coupled with relatively high rates of nucleotide substitution and selection pressure in the *Y. ruckeri* genome (Table 1). However, after the sharp decline observed in the effective size of *Y. ruckeri* population between 1975 and 2000, which coincided with the successful period of the commercial vaccine, a soaring increase in the genetic diversity of *Y. ruckeri* was registered after just six years from the maximum population decline. This unexpected finding is associated to the emergence of virulent biotype 2 *Y. ruckeri* strains causing of ERM outbreaks in vaccinated fish[5,6,10,30]. We have not detected evidences of a stabilizing period in the population between the decline period, occurred from 1975 and 2000, and the early increase observed from 2000. This

dynamic can support the theory that during rapid demographic expansions, both genetic drift and natural selection will be reduced, thus leading to an increase, at least transiently, in the number of segregating mutations in the population[28].

Discussion

We have revealed the evolutionary history of *Y. ruckeri* at unprecedented detail reconstructing the ancestral divergence and geographical spread over time of this pathogen. The high short-term evolutionary rate in *Y. ruckeri* enabled the estimation of divergence times and the analyses of past changes in population size on the basis of a time-structured serial DNA sequence samples. The inferred pattern of *Y. ruckeri* spread among countries corroborated that USA was the initial source for the ERM dispersion. Here we have clearly demonstrated instances of gene flow between regions in North America and Europe, facilitated by aquaculture expansion. This study also revealed that the non-motile *Y. ruckeri* strains, associated to the early 1995 and posterior 2001 ERM epizootics in UK[5], have arisen from isolates already existing within UK since previous epizootics (1980 and 1995, respectively). Similar scenario may explain the emergence of the biotype 2/serotype O1b *Y. ruckeri* strains causing epizooties between 2003 and 2005 in USA[8]. On the other hand, the lack of statically supported epidemiological linkage between USA and Portugal or Peru, as well as Finland with any other areas, suggests the independent emergence of biotype 2 *Y. ruckeri* lineages in the different continents. The existence of different *Y. ruckeri* lineages within each country indicates that aquaculture have driven the *in situ* evolution of this bacterial pathogen, which may explain the maintenance of ERM in the world.

It is intriguing that the overall age of *Y. ruckeri* emergence has been calculated

within the last 500 years (lowest limit detected among all the TMRCAs). However, since this time, *Y. ruckeri* has differentiated into seven antigenically distinct groups[27]. It is therefore tempting to speculate that the high rate of evolutionary change determined for *Y. ruckeri*, may in part explain the great antigenic diversity achieved by this pathogen and, in turn, the failures in efficacy of the commercial vaccine developed against the ERM almost forty years ago. Furthermore, this study showed that there is an increasing number of BT2 and serotype O1b (in USA, UK and Chile) strains that could continue to cause problems in the future. There is a possibility that non-motile *Y. ruckeri* infections are increasing in prevalence due to the removal of motile isolates from the environment through vaccination[31]. The observed change from motile to high virulent non-motile *Y. ruckeri* isolates being recovered from ERM outbreaks could be because vaccine induced strain replacement[32]. The presence of several genetically distinct bacterial populations in one area might favour virulence, if the virulent strains have a competitive advantage[31]. Thus, the current increase of biotype 2 *Y. ruckeri* cases worldwide, as well as the emergence of virulent motile isolates belonging to serotype O1b in South America could be understood as consequence of the maintenance of intensive aquaculture procedures. Our findings demonstrate the influence of a sustainable human activity on the evolution of *Y. ruckeri*, and may constitute a useful model for the study of the anthropogenic factors in the evolution of others human and animal pathogens.

METHODS SUMMARY

We have development a multilocus sequence-typing (MLST) scheme for *Y. ruckeri* in previous studies[12]. For this study, DNA partial sequences of six housekeeping genes including *gln*A (glutamine synthetase), *gyr*B (DNA gyrase B subunit), *rec*A (DNA repair and recombination), Y-HSP60 (60-KDs heat shock protein), *dna*J (heat shock protein 40) and *thr*A (aspartokinase-homoserine dehydrogenase), were downloaded from the *Yersinia ruckeri* MLST database hosted on publmlst.org and freely available from http://pubmlst.org/yruckeri/. Data sets consisted for each HK gene, of 103 *Y. ruckeri* sequences geographically diverse sampled over a 44-year period (1965-2009) and belonging different serotypes, biotype and diverse host/source of isolation as described in the *Yersinia ruckeri* MLST database (see Supplementary Table 1). The concatenated sequences (2,786 bp) for each isolate were also obtained and analyzed. A Bayesian Monte-Carlo Markov chain (MCMC) approach (Drummond and Rambaut, 2007) available in the Beast version 1.7.2 package[33] was used to determine the rate of nucleotide substitution per site and the time to the most recent common ancestor (TMRCA) in each HK gene and concatenated sequence. Bayesian skyline plot coalescent model in all cases was also employed to describe the complex population dynamics and inferring demographic processes[29]. To quantify the rate at which *Y. ruckeri* linages move among sampled geographical locations a discretized spatial diffusion model[26] was using employed the BEAST software v1.7.2. The maximum clade credibility (MCC) tree obtained was visualized by using the software Spread-phy v 1.0.4[34]. This software was also employed to determine the statistical significance for the migration rates among the locations using Bayes factor[35], and to export the MCC tree to .xml format.

Finally, we visualized this information in Google Earth, as a tree that grows over time, seeding locations each time an ancestral node is inferred to exist at a different location. More detailed descriptions of all methods used in this paper can be found in the Supplementary Methods.

REFERENCES

1. Knowless LL and Madison WP (2002) Statistical phylogeography. Mol Ecol 11: 2623–2635.
2. Griffiths RC and Tavaré S (1994) Ancestral Inference in Population Genetics. Statist Sci 9: 307-319.
3. Lemey P, Rambaut A, Welch JJ and Suchard MA (2010) Phylogeography takes a relaxed random walk in continuous space and time. Mol Biol Evol 27: 1877-1885.
4. Rambaut A and Bromham L (1998) Estimating Divergence Dates from Molecular Sequences. Mol Biol Evol. 15: 442–448.
5. Austin DA, Robertson PAW and Austin B (2003) Recovery of a new biogroup of Yersinia ruckeri from diseased rainbow trout (*Oncorhynchus mykiss,* Walbaum). Syst Appl Microbiol 26: 127–131.
6. Fouz B, Zarza C and Amaro C (2006) First description of non-motile *Yersinia ruckeri* serovar I strains causing disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum), cultured in Spain. J Fish Dis 29: 339–346.
7. Wheeler RW, Davies RL, Dalsgaard I, Garcia J, Welch TJ, Wagley S, et al. (2009) *Yersinia* ruckeri biotype 2 isolates from mainland Europe and the UK likely represent different clonal groups. Dis Aquat Organ 84: 25–33.

8. Arias CR, Olivares-Fuster O and Hayden K (2007) First report of *Yersinia ruckeri* biotype 2 in the USA. J Aquat Anim Health 19: 35–40.
9. Romalde JL, Planas E, Sotelo JM and Toranzo AE (2003) First description of *Yersinia ruckeri* serotype O2 in Spain. Bull Eur Assoc Fish Pathol 23:135–138.
10. Bastardo A, Bohle H, Ravelo C, Toranzo AE and Romalde JL (2011) Serological and molecular heterogeneity among *Yersinia ruckeri* strains isolated from farmed Atlantic salmon *Salmo salar* in Chile. Dis Aquat Organ 93: 207–214.
11. Bridle A, Nosworthy E, Polinsky M and Nowak B (2011) Evidence of an antimicrobial-immunomodulatory role of Atlantic salmon cathelicidins during infection with *Yersinia ruckeri.* PLoS One 6:e23417.
12. Bastardo A, Ravelo C and Romalde JL (2012) Multilocus sequence typing reveals high genetic diversity and epidemic population structure for the fish pathogen *Yersinia ruckeri*. Environ Microbiol 14: 1888-1897.
13. Pérez-Losada M, Crandall KA, Zenilman J and Viscidi RP (2007) Temporal trends in gonococcal population genetics in a high prevalence urban community. Infect Genet Evol 7: 271–278.
14. Gilbert MT, Rambaut A, Wlasiuk G, Spira TJ, Pitchenik AE and Worobey M (2007) The emergence of HIV/AIDS in the Americas and beyond. Proc Natl Acad Sci USA 104: 18566–70.
15. Falush D, Kraft C, Taylor NS, Correa P, Fox JG, et al. (2001) Recombination and mutation during long-term gastric colonization by *Helicobacter pylori*: estimates of clock rates, recombination size, and minimal age. Proc Natl Acad Sci U S A 98: 15056–15061.
16. Achtman M and Wagner M (2008) Microbial diversity and the genetic nature

of microbial species. Nat Rev Microbiol. 6: 431–440.

17. Wilson DJ, Gabriel E, Leatherbarrow AJ, Cheesbrough J, Gee S, et al. (2009) Rapid evolution and the importance of recombination to the gastroenteric pathogen Campylobacter jejuni. Mol Biol Evol 26: 385–397.
18. Drummond AJ and Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7: 214
19. Drummond AJ, Ho SY, Phillips MJ and Rambaut A (2006) Relaxed phylogenetics and dating with confidence. PLoS Biol. 4: e88
20. Guttman DS and Dykhuizen DE (1994) Clonal divergence in *Escherichia coli* as a result of recombination, not mutation. Science 266: 1380–1383.
21. O'Neill AJ, Cove JH and Chopra I (2001) Mutation frequencies for resistance to fusidic acid and rifampicin in *Staphylococcus aureus*. J Antimicrob Chemother 47: 647–650.
22. Al Mamun AAM (2007) Elevated expression of DNA polymerase II increases spontaneous mutagenesis in Escherichia coli. Mutat Res 625: 29–39.
23. Nubel U, Dordel J, Kurt K, Strommenger B, Westh H, et al. (2010) A Timescale for Evolution, Population Expansion, and Spatial Spread of an Emerging Clone of Methicillin-Resistant *Staphylococcus aureus*. PLoS Path 6: e1000855.
24. Morelli G, Song YJ, Mazzoni CJ, Eppinger M, Roumagnac P, Wagner DM., Feldkamp M, Kusecek B., Vogler AJ., Li Y, Cui Y., Thomson NR., Jombart T, Leblois R, Lichtner P, Rahalison L, Petersen JM, Balloux F, Keim P, Wirth T, Ravel J, Yang R, Carniel E and Achtman M (2010) *Yersinia pestis* genome sequencing identifies patterns of global phylogenetic diversity. Nat Genet 42: 1140–1143.

25. Feng L, Reeves PR, Lan R, Ren Y, Gao C, et al. (2008) A recalibrated molecular clock and independent origins for the cholera pandemic clones. PLoS ONE 3: e4053.
26. Lemey P, Rambaut A, Drummond AJ, and Suchard MA (2009) Bayesian phylogeography finds its roots. PLoS Comput. Biol. 5:e1000520.
27. Romalde JL, Margariños B, Barja JL and Toranzo AE (1993) Antigenic and molecular characterization of *Yersinia ruckeri*. Proposal for a new intraspecies classification. Syst Appl Microbiol 16: 411–419.
28. Hahn MW, Rausher MD and Cunningham CW (2002) Distinguishing between selection and population expansion in an experimental lineage of bacteriophage T7. Genetics 161: 11–20.
29. Drummond AJ, Rambaut A, Shapiro B and Pybus OG (2005) Bayesian Coalescent Inference of Past Population Dynamics from Molecular Sequences. Mol Biol Evol 22: 1185–1192.
30. Welch TJ, Verner-Jeffreys DW, Dalsgaard I, Wiklund T, Evenhuis JP, et al. (2011) Independent emergence of *Yersinia ruckeri* biotype 2 in the United States and Europe. Appl Environ Microbiol 77: 3493–3499.
31. Pulkkinen K., Suomalainen L.-R., Read A.F., Ebert D., Rintamäki P., et al. (2010) Intensive fish farming and the evolution of pathogen virulence: the case of columnaris disease in Finland. Proc R Soc B 277:593–600.
32. Martcheva M, Bolker BM and Holt RD (2008) Vaccine-induced pathogen strain replacement: What are the mechanisms? J R Soc Interface 5 :3–13.
33. Drummond C. S., Eastwood R. J., Miotto S. T. S. and Hughes, C. E. **(**2012) Multiple continental radiations and correlates of diversification in *Lupinus* (Leguminosae): testing for key innovations with incomplete taxon sampling. Syst Biol doi: 10.1093/sysbio/syr126

34. Bielejec F, Rambaut A, Suchard MA and Lemey P (2011) SPREAD: spatial phylogenetic reconstruction of evolutionary dynamics. Bioinformatics 27: 2910–2912.
35. Kass RE and Raftery AE (1995) Bayes factors. Journal of American Statistical Association 90: 773–795.

Table 1. Evolutionary parameters in *Y. ruckeri* estimated of by Bayesian analyses. Mean: mean value (substitutions/site/year), HPD: highest posterior density values.

Data set	Mean substitution rate	95% HPD (lower-upper)
*dna*J	1.9×10^{-5}	3.4×10^{-4} –3.7×10^{-5}
*gyr*B	2.6×10^{-5}	1.8×10^{-4} –4.8×10^{-5}
*gln*A	2.0×10^{-5}	2.2×10^{-4}–6.2×10^{-5}
Y-HSP60	1.6×10^{-5}	9.0×10^{-4}–5.1×10^{-5}
*rec*A	1.9×10^{-5}	2.1×10^{-4}–6.0×10^{-5}
*thr*A	1.3×10^{-5}	1.1×10^{-4}–8.4×10^{-5}
Overall	2.8×10^{-5}	4.2×10^{-4}–7.4×10^{-5}

Figure legends

Figure 1. Bayesian maximum clade credibility (MCC) phylogeny for concatenated sequences of *Yersinia ruckeri* with continuous-time Markov chain (CTMC) spatial reconstruction. Taxon labels include strain designation, country of isolation, and year of isolation. Terminal branches of the tree were colored according to the sample location of the taxon at the tip (see color codes in the histogram in upper left insert). Internal branches are colored according to the most probable location state of their descendent nodes. Nodes with posterior probabilities of > 0.6 have been labeled accordingly (in black). The probabilities of location states of selected nodes are shown (as percentages) in red: high probability from USA origin, and blue from UK origin. To the histogram inserted shows the posterior probabilities for the locations of the root nodes. A colored arrow indicates the divergence of the BT2 and more important resistant vaccine emergent sequences as their origin.

Figure 2. Temporal dynamics of spatial *Yersinia ruckeri* diffusion. Snapshots of the dispersal pattern among countries for 1960, 1976, 1978, 1987, 1997, and 2009 were provided. Lines between locations represent branches in the maximum clade credibility (MCC) tree along which the relevant location transition occurs. Location circles diameters are proportional to square root of the number of MCC branches maintaining a particular location state at each time-point. The yellow-red color gradient indicates the relative age of the transition (older-recent).

Figure 3. Bayes factor test for significant non-zero rates for the *Yersinia ruckeri* diffusion among-countries analysis. Only rates supported by a BF> 3 are

indicated. The colors of the lines represent the relative strength by with the rates are supported (from dark red= weak to light red= strong).

Figure 4. Bayesian skyline plot showing changes in effective population size (y-axis) of *Yersinia ruckeri* through time (black line), estimated from the concatenated data set. The blue shaded area represents 95% confidence intervals.

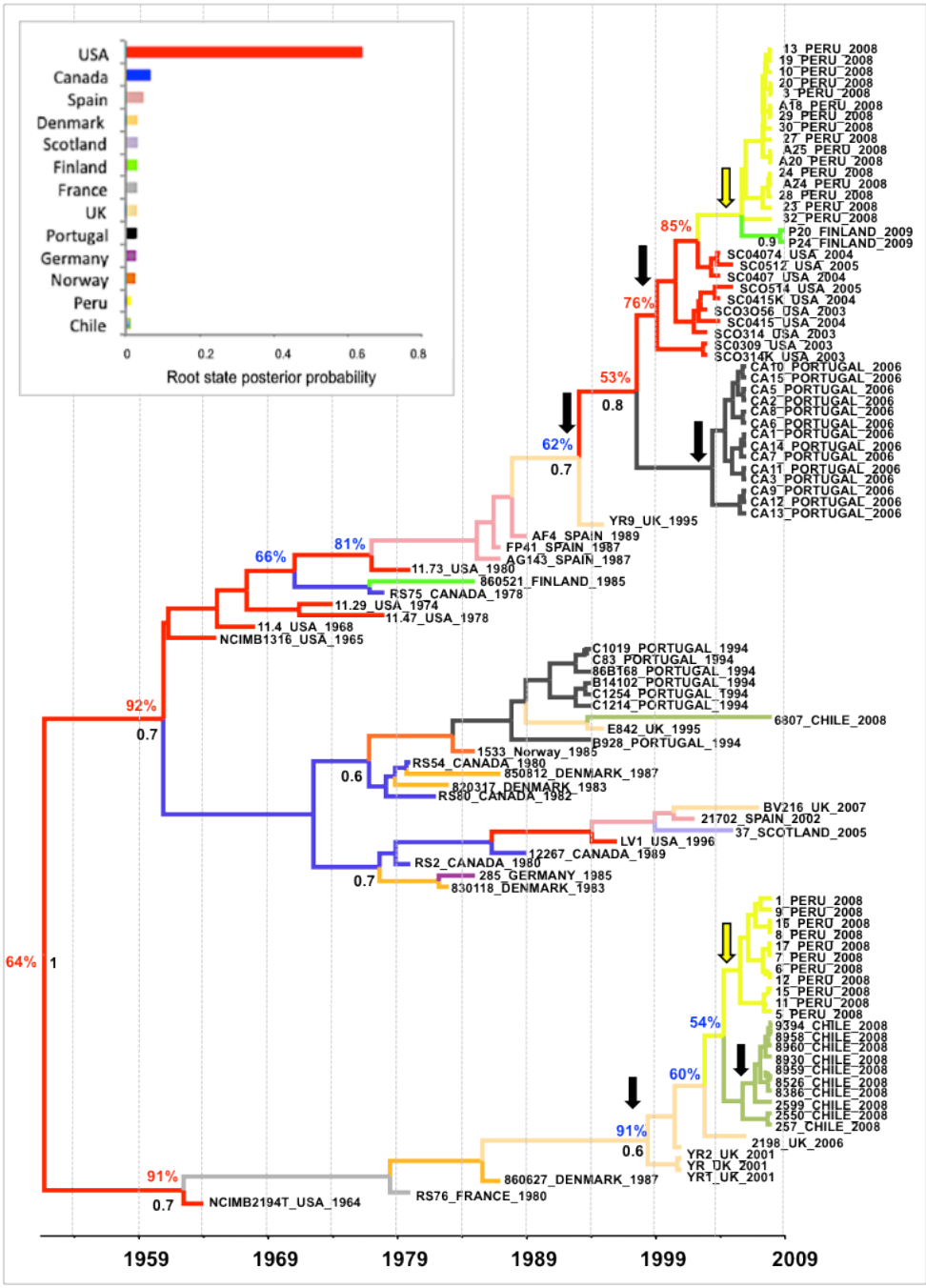


Figure 1. Bastardo et al.

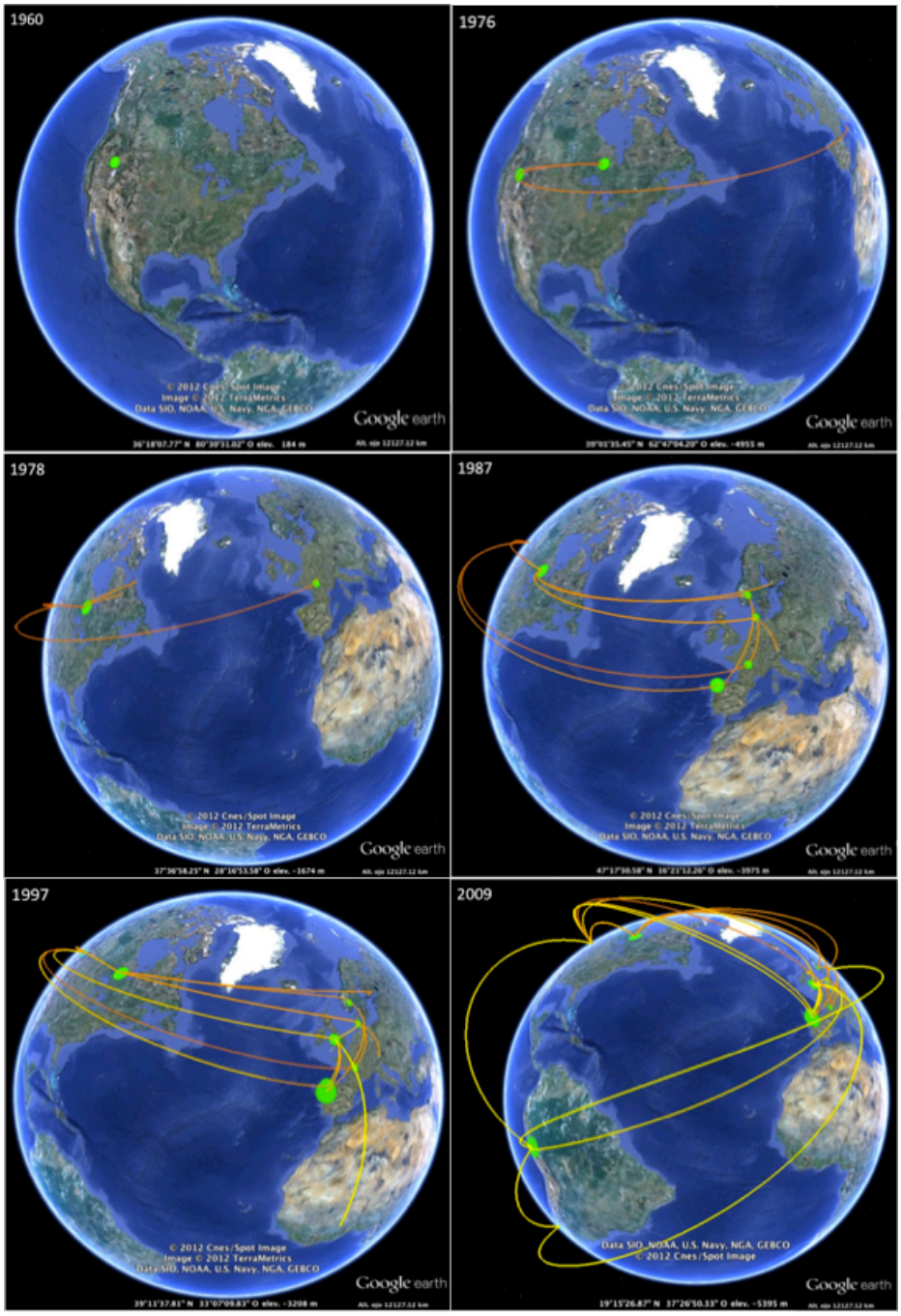


Figure 2. Bastardo et al.

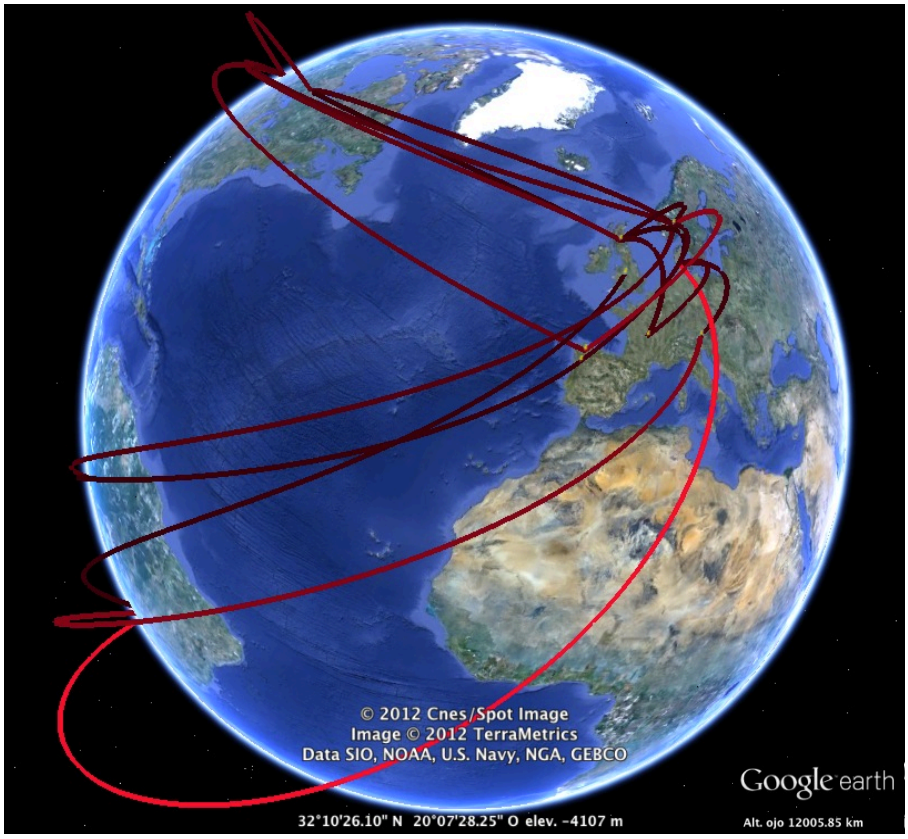


Figure 3. Bastardo et al.

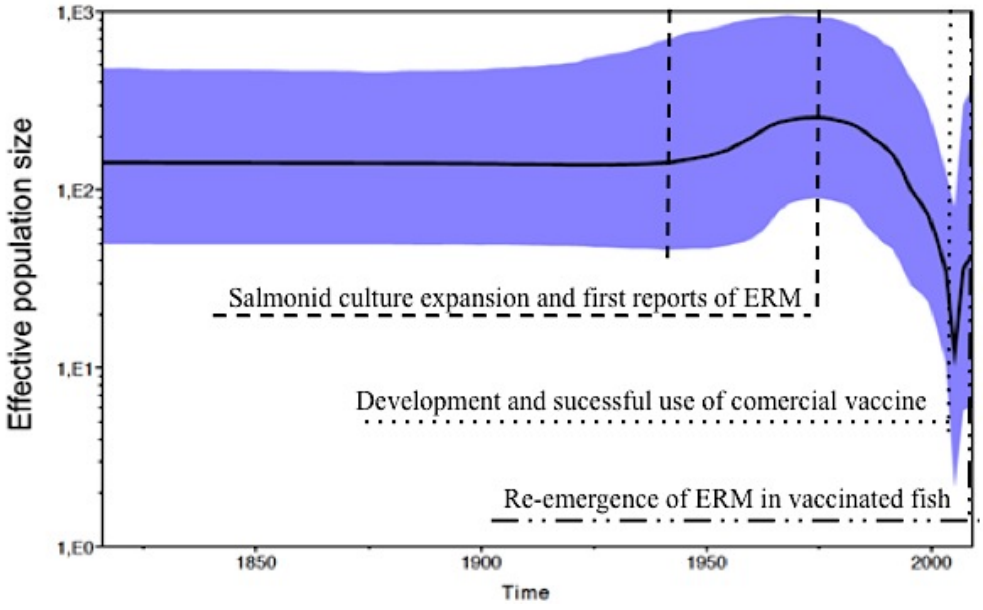


Figure 4. Bastardo et al.

AQUACULTURE DRIVES EVOLUTION OF BACTERIAL PATHOGENS: THE *Yersinia ruckeri* TALE.

SUPPLEMENTARY MATERIAL

METHODS

Bayesian MCMC analyses.

A Bayesian Monte-Carlo Markov chain (MCMC) approach[18] available in the Beast version 1.7.2 package (http://beast.bio.ed.ac.uk/) was used to determine the rate of nucleotide substitution per site and the time to the most recent common ancestor (TMRCA) for each data set. In each case relaxed (uncorrelated lognormal) molecular clocks[19] and a Hasegawa-Kishino-Yano (HKY) nucleotide substitution model[36] were used with a discretized gamma distribution to model rate heterogeneity among sites[37] and to account the variation in the rates of evolution. The uncorrelated lognormal molecular clock was selected over a strict clock based on the preliminary results obtained of the linear regression methods implemented in Path-O-Gen version 1.3 (http://tree.bio.ed.ac.uk/), which indicated deviation from strict clock. Bayesian skyline plot (BSP) coalescent model in all cases was also employed to describe the complex population dynamics and inferring demographic processes[29]. For each dataset, three to five independent Bayesian MCMC runs were conducted for sufficient time to achieve convergence (assessed using the Tracer version 1.5 program (http://tree.bio.ed.ac.uk/software/tracer/) with uncertainty in parameter estimates reflected in values of the 95% highest probability density (HPD). The results of the multiple runs were then combined using LogCombiner version 1.4.7 2 (http://beast.bio.ed.ac.uk). To compute the maximum clade credibility (MCC) tree

from all the plausible trees the TreeAnnotator program version 1.5.2 (http://beast.bio.ed.ac.uk) was used, removing the first 10% trees as burn-in, and to visualize the annotated MCC tree the software FigTree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/) was employed.

Phylogeographic reconstruction through time and space

To quantify the rate at which *Y. ruckeri* linages move among sampled geographical locations a discretized spatial diffusion model[26] was employed using the BEAST software v1.7.2. Analyses were carried out using the identical above conditions for the substitution model, molecular clock model, and tree prior model. To achieve statistical efficiency, a Bayesian stochastic search variable selection (BSSVS) procedure was also employed[26] with a truncated Poisson prior with a mean of log 2 and an offset of K-1, where K represents the number of discretized location states. To fit this temporal-spatial process model simultaneously with well-established models of sequence evolution in a Bayesian genealogical approach the software package BEAST was used combined with the general phylogenetic likelihood evaluation library BEAGLE[38].

For phylogeographic analyses each sequence was assigned a specific "character state" based on its geographic origin. Although the analysis considers 30 locations from 13 countries different, only movement among countries (13 character states, i.e., USA, UK, Chile, Peru, Portugal, Canada, Finland, Spain, Denmark, France, Germany, Scotland, and Norway) we considered. Where two discrete locations were grouped together, the longitude and latitude used were those of the midpoint of the line connecting them. Where more than two locations were grouped, the latitude and longitude of the centroid of the polygon defined by them were used. TreeAnnotator was used to summarize these trees using a representative

clustering pattern (MCC tree) and annotate these clusters with the most probable location states.

The MCC tree was visualized by using the software Spread-phy v 1.0.4 (http://www.kuleuven.be/aidslab/phylogeography/SPREAD.html)[34] and exported to Google Earth, to visualize this information as a tree that grows over time, seeding locations each time an ancestral node is inferred to exist at a different location. The Spread-phy v1.0.4 software was also employed to analyze the statistical significance for the migration rates among the location states using Bayes factor[35].

36. Hasegawa M, Kishino H and Yano TJ (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. Mol Evol 22:160–174.
37. Yang Z (1994) Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: Approximate methods. J Mol Evol 39: 306–314.
38. Suchard MA and Rambaut A (2009) Many-core algorithms for statistical phylogenetics. Bioinformatics 25: 1370–1376.

Supplementary Table 1

Table S1. Estimated time of the most common ancestors for representative and re-emergent *Yersinia ruckeri* strains for each housekeeping gene and concatenated sequences analyzed.

Y. ruckeri strain	Date MRCA[a] existed: mean (95% HPD[b])						
	*dna*J	*gln*A	*gyr*B	Y-HSP60	*rec*A	*thr*A	Concatenated
NCIMB2194[T]	1943 (1920-1964)	1939 (1876-1957)	1957 (1949-1960)	1960 (1960-1961)	1942 (1927-1961)	1956 (1918-1963)	1954 (1944-1964)
Representative O-serotype[a]							
11.4 (O1a)	1954 (1961-1920)	1929 (1867-1959)	1957 (1949-1960)	1960 (1960-1961)	1959 (1958-1962)	1949 (1910-1965)	1937 (1889-1960)
1533 (O1b)	1969 (1958-1980)	1984 (1983-1984)	1978 (1968-1983)	1977 (1974-1977)	1981 (1975-1983)	1981 (1980-1985)	1979 (1914-1985)
RS2 (O2a)	1960 (1933-1974)	1961 (1961-1971)	1972 (1966-1978)	1976 (1975-1976)	1968 (1964-1968)	1974 (1965-1978)	1917 (1842-2004)
11.29 (O1a)	1947 (1907-1972)	1951 (1892-1971)	1967 (1959-1973)	1970 (1970-1973)	1959 (1958-1962)	1959 (1919-1968)	1927 (1887-1955)
11.47 (O3)	1957 (1953-1965)	1951 (1892-1973)	1967 (1959-1973)	1973 (1971-1978)	1974 (1967-1978)	1963 (1945-1974)	1957 (1938-1975)
11.73 (O4)	1967 (1960- 1978)	1962 (1961-1971)	1976 (1967-1980)	1977 (1974-1977)	1916 (1840-1957)	1974 (1965-19789	1957 (1938-1968)
Emerging BT2 isolates[b]							
UK 1995 (O1a)	1979 (1974-1985)	1988 (1984-1989)	1986 (1981-1993)	1992 (1988-1995)	1990 (1989-1990)	1992 (1986-1995)	1980 (1962-1987)
UK 2001 (O1a)	1977 (1974-1985)	1992 (1985-1994)	1992 (1992-1993)	1985 (1980-1987)	1990 (1989-1990)	1987 (1987-1995)	1995 (1995-1987)
USA 2003 (O1b)	1994 (1983-1997)	1980 (1980-1987)	1996 (1992-1997)	1989 (1987-1989)	1988 (1987-1988)	1987 (1987-1992)	1970 (1951-1978)
Portugal 2006 (O1a)	2002 (1998-2002)	1997 (1995-1997)	1998 (1997-1998)	1997 (1995-1998)	1987 (1986-1987)	2000 (1998-2004)	2005 (2004-2005)
Peru 2008 (O1a)	2004 (2004-2007)	1999 (1987-1999)	2002 (1995-2003)	2005 (2004-2005)	2002 (2002-2003)	2003 (200-2003)	2003 (1994-2001)
Finland 2009 (O1a)	2006 (2005-2008)	2007 (2006-2007)	1996 (1995-1997)	2005 (2004-2005)	2001 (2001-2002)	2003 (2003-2004)	2006 (2004-2006)
Emerging BT1 isolates[c]							
Chile 2008 (O1b)	1988 (1991-1994)	2000 (1999-2005)	2001 (1998-2000)	1994 (1993-1994)	1998 (1997-2003)	1993 (1987-1994)	1983 (1971-1987)
Spain 2002 (O2b)	1976 (1971-1983)	2000 (1997-2001)	2001 (2000-1002)	1993 (1993-1994)	1998 (1998-1999)	1995 (1994-1998)	1970 (1940-1983)
Root MCC tree	1639 (1639-1861)	1860 (1723-1941)	1812 (1608-1937)	1891 (1757-1955)	1685 (1380-1876)	1865 (1707-1945)	1557 (1501-1816)

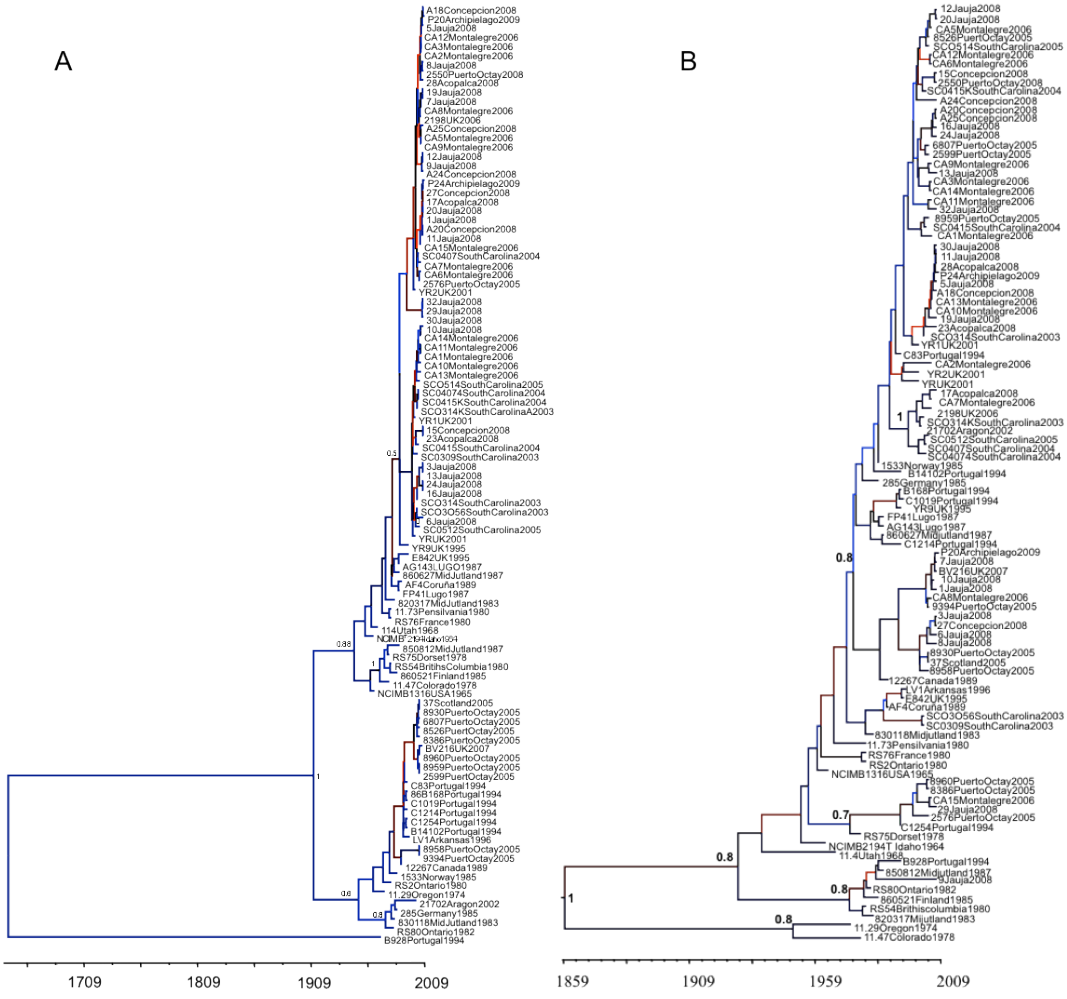
[a]MRCA: Most recent common ancestor; [b] 95% HPD: highest posterior density intervals (lower-upper) values at 95% credibility.

Supplementary Table 2

Table S2. Bayes factor test for to determine the significance of non-zero rates of among-country analysis of *Yersinia ruckeri.*

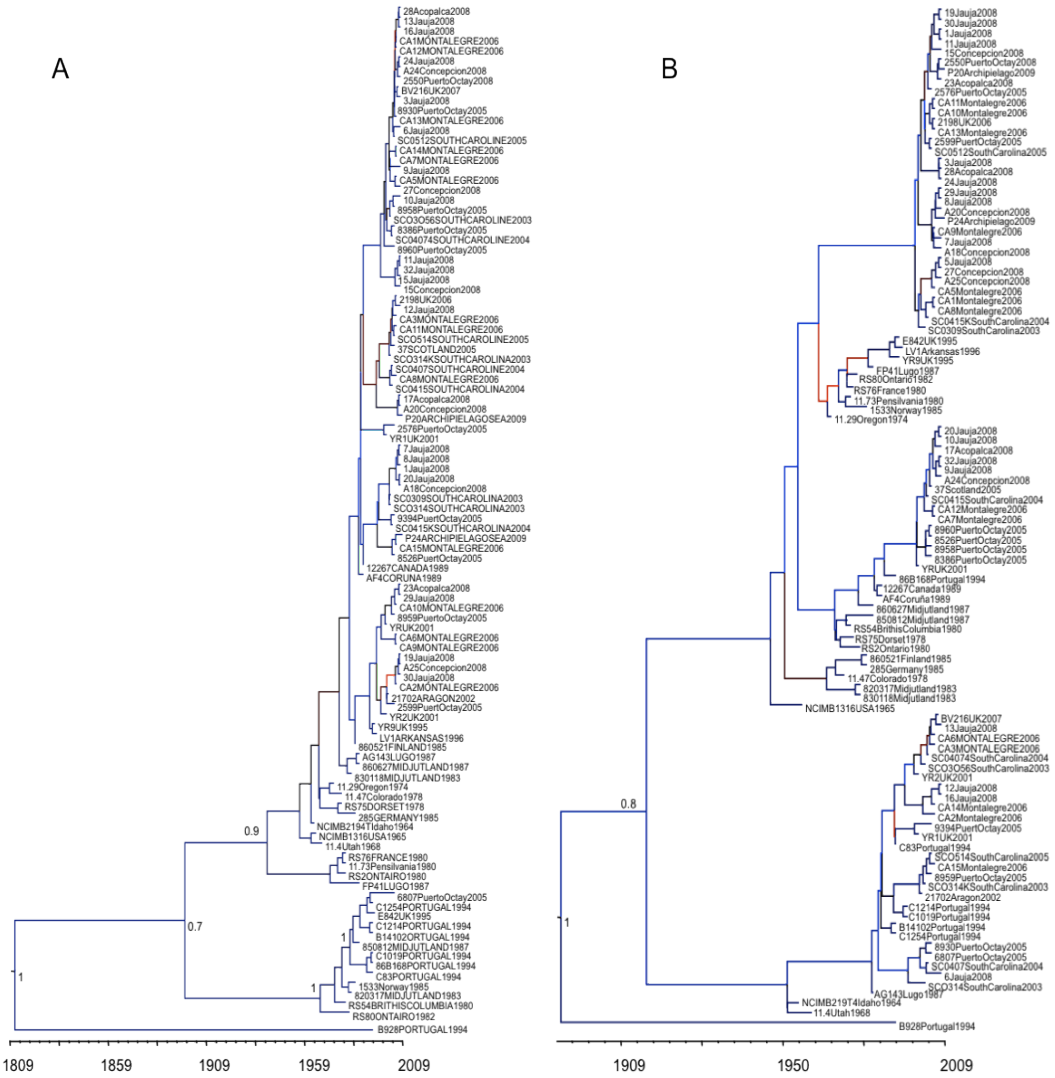
Bayes factor	Linkage that support	
21.8	Denmark	Chile
9.9	Norway	Spain
9.9	USA	Spain
9.2	Germany	Chile
7.4	USA	Scotland
7.5	USA	Canada
5.9	USA	Norway
5.8	Norway	Peru
5.7	Denmark	France
5.6	Canada	Norway
5.4	France	Scotland
5.4	Norway	Portugal
5.3	Denmark	Germany
5.2	Norway	Scotland
5.1	Portugal	Peru
5.1	UK	Chile
4.1	Denmark	Scotland
3.0	Canada	Scotland

Supplementary figure 1. Maximum clade credibility tree of partial gene sequences of *Yersinia ruckeri*. A) *dna*J gene; B) *gln*A gene. All tip times on the tree correspond to the year of sampling (as reflected in the timescale on the x axis). Posterior probability values (> 0.6) a measure of clustering support are shown for key nodes. Color-coding of branches indicate different molecular rates: red colored branches correspond to higher molecular rates, while blue correspond to slower molecular rates. The tree was rooted on the assumption of a relaxed molecular clock. Nodes correspond to mean of time of the most common ancestors (TMRCAs).



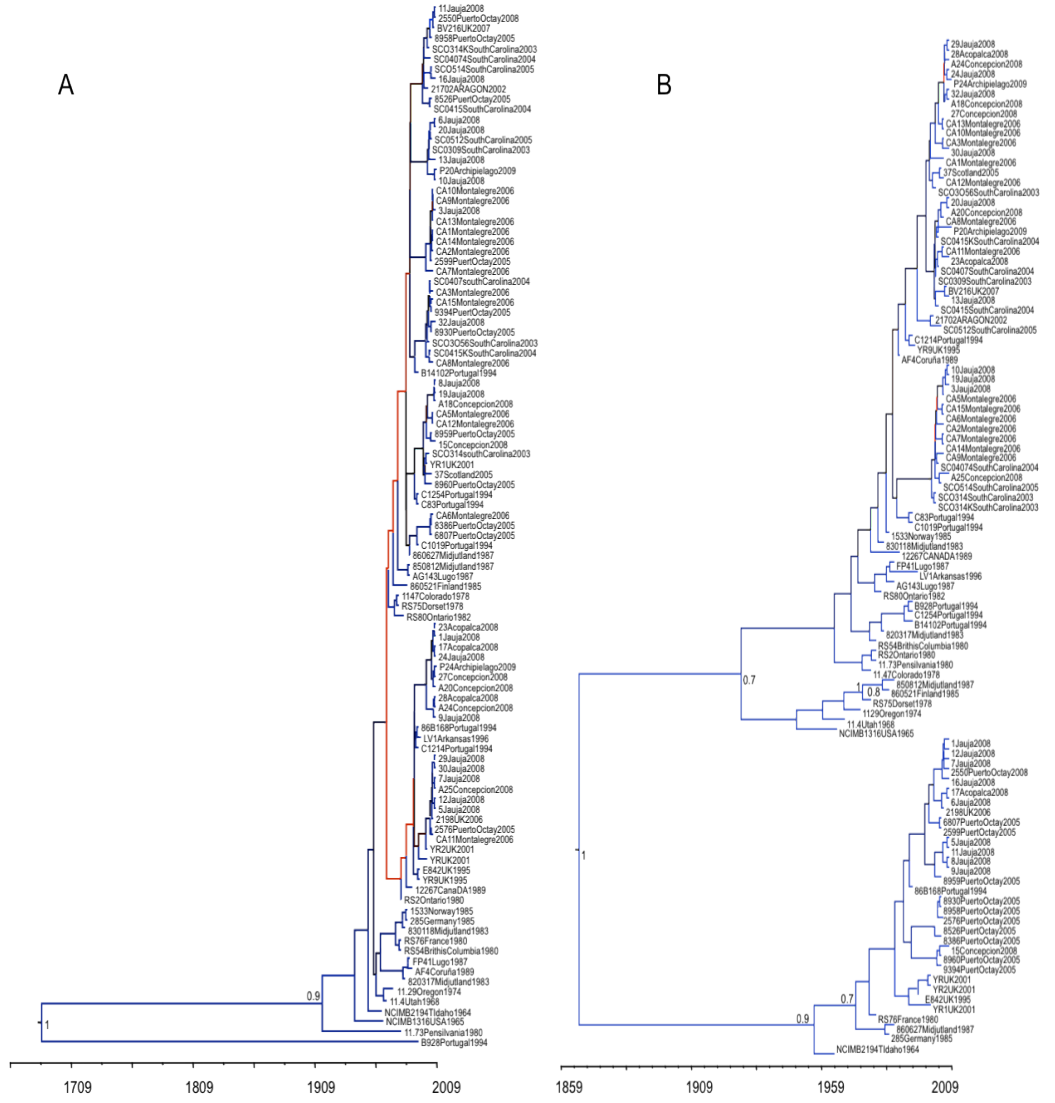
Supplementary figure 1. Bastardo et al.

Supplementary figure 2. Maximum clade credibility tree of partial gene sequences of *Y. ruckeri*. A) *gyr*B gene; B) Y-HSP60 gene. All tip times on the tree correspond to the year of sampling (as reflected in the timescale on the x axis). Posterior probability values (> 0.6) a measure of clustering support are shown for key nodes. Color-coding of branches indicate different molecular rates: red colored branches correspond to higher molecular rates, while blue correspond to slower molecular rates. The tree was rooted on the assumption of a relaxed molecular clock. Nodes correspond to mean of time of the most common ancestors (TMRCAs).



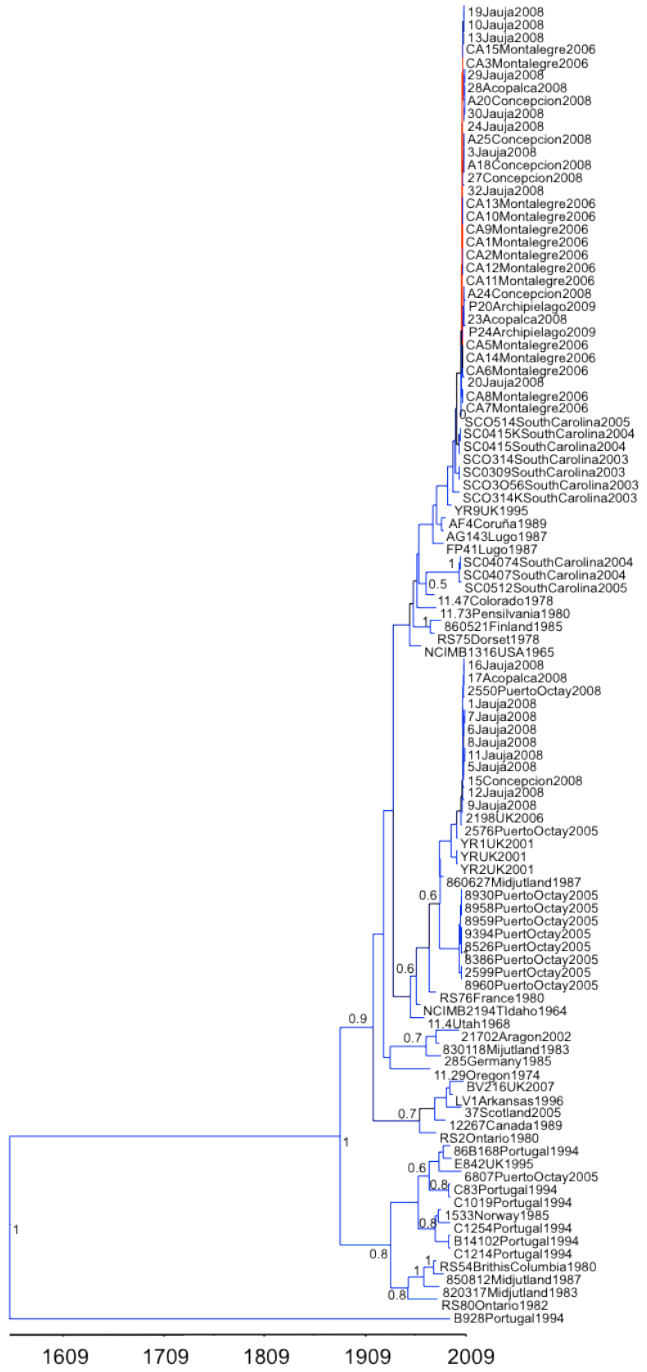
Supplementary figure 2. Bastardo et al.

Supplementary figure 3. Maximum clade credibility tree of partial gene sequences of *Y. ruckeri*. A) *rec*A gene; B) *thr*A gene. All tip times on the tree correspond to the year of sampling (as reflected in the timescale on the x axis). Posterior probability values (> 0.6) a measure of clustering support are shown for key nodes. Color-coding of branches indicate different molecular rates: red colored branches correspond to higher molecular rates, while blue correspond to slower molecular rates. The tree was rooted on the assumption of a relaxed molecular clock. Nodes correspond to mean of time of the most common ancestors (TMRCAs).



Supplementary figure 3. Bastardo et al.

Supplementary figure 4. Dated phylogeny of the concatenated sequence of six HK genes of *Y. ruckeri.* The tree is scaled to time (horizontal axis) and was generated using the Hasegawa-Kishino-Yano (HKY) codon substitution model and uncorrelated relaxed clock model. Posterior probability values (> 0.6) measures of clustering support are shown for key nodes. Color-coding of branches indicate different molecular rates: red colored branches correspond to higher molecular rates, while blue correspond to slower molecular rates. Nodes correspond to mean of time of the most common ancestors (TMRCAs).



Supplementary figure 4. Bastardo et al.

Supplementary video*: Temporal dynamic of spatial *Y. ruckeri* diffusion visualized using Google Earth. Branches show traversing arcs between reconstructed locations over the time they span.

*** El video citado como material suplementario en el texto está disponible en la versión digital (CD-ROM) que acompaña esta memoria.**

Supplementary video. Bastardo et al.

6. CAPÍTULO IV:

Detección y cuantificación de *Yersinia ruckeri* mediante real-time PCR.

Artículo:

Highly sensitive detection and quantification of the pathogen *Yersinia ruckeri* in fish tissues by using real-time PCR

Autores:

Asmine Bastardo, Carmen Ravelo and Jesús L. Romalde

Referencia:

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Indices de calidad:

Factor de impacto JCR: 3,425

Categoría "Biotechnology and Applied Microbiology": Q1

Resumen:

Yersinia ruckeri es el agente causal de enfermedad entérica de la boca roja (ERM) y uno de los principales patógenos bacterianos causantes de pérdidas en la acuicultura de salmónidos. Debido a que recientemente se han descrito fallas en la protección de la vacunación contra la ERM, atribuídas principalmente a la aparición de cepas *Y. ruckeri* del biotipo 2, se hace necesario el desarrollo de métodos de detección rápidos reproducibles y sensibles. En este estudio, se diseño y optimizó una reacción de la polimerasa en tiempo real en cadena (PCR) con un set de cebador/sonda basado en el gen de la proteína recombinasa A (*rec*A) para mejorar la detección de *Y. ruckeri*. El set cebador/sonda demostró tener una especificidad de 100% y una sensibilidad analítica de 1,8 ag μl^{-1}, equivalente a 1,7 unidades formadoras de colonias (CFU) ml^{-1}, en el DNA purificado; de 3,4 UFC

g^{-1} en los tejidos inoculados hígado, riñón y bazo, y de 0,34 UFC/100 μl^{-1} en sangre. El ensayo fue altamente reproducible con bajos valores de coeficiente de variación intra e inter experimentos de 2,9% y 9,5%, respectivamente. Después de la optimización, el ensayo se utilizó para detectar cambios en la carga bacteriana durante una infección experimental. Varios ejemplares de trucha arcoíris (*Oncorhynchus mykiss*) se expusieron separadamente a dos cepas de *Y. ruckeri* (biotipo 1 y biotipo 2) mediante inoculación intraperitoneal. Los órganos internos (hígado, riñón, bazo) y la sangre se obtuvieron a partir de peces muertos cada día durante 15 días, para cuantificar copias del DNA del patógeno por gramo de tejido. Los resultados demostraron la eficacia de este ensayo de PCR en tiempo real para cuantificar células de *Y. ruckeri* en los tejidos de los peces, y también confirmaron este ensayo como un método no letal para la detección de este patógeno en muestras de sangre.

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METHODS AND PROTOCOLS

Highly sensitive detection and quantification of the pathogen *Yersinia ruckeri* in fish tissues by using real-time PCR

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Abstract *Yersinia ruckeri* is the causative agent of enteric redmouth diseases (ERM) and one of the major bacterial pathogens causing losses in salmonid aquaculture. Since recent ERM vaccine breakdowns have been described mostly attributed to emergence of *Y. ruckeri* biotype 2 strains, rapid, reproducible, and sensitive methods for detection are needed. In this study, a real-time polymerase chain reaction (PCR) primer/probe set based on recombination protein A (*rec*A) gene was designed and optimized to improve the detection of *Y. ruckeri*. The primer/probe set proved to have a 100 % analytical specificity and a sensitivity of 1.8 ag μl^{-1}, equivalent to 1.7 colony-forming units (CFU) ml^{-1}, for purified DNA, 3.4 CFU g^{-1} for seeded liver, kidney, and spleen tissues, and 0.34 CFU/100 μl^{-1} for seeded blood, respectively. The assay was highly reproducible with low variation coefficient values for intra- and inter-run experiments (2.9 % and 9.5 %, respectively). Following optimization, the assay was used to detect changes in the bacterial load during experimental infection. Rainbow trout (*Onchorhynchus mykiss*) were exposed to two strains of *Y. ruckeri* (biotype 1 and biotype 2) by intraperitoneal inoculation. Internal organs (liver, kidney, spleen) and blood were biopsied from dead fish daily for 15 days to quantify copies of pathogen DNA per gram of tissue. The findings showed the efficacy of this real-time PCR assay to quantify *Y. ruckeri* cells in the fish tissues and also confirmed this assay as a non-lethal method for the detection of this pathogen in blood samples.

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Keywords Real-time PCR · *Yersinia ruckeri* · Enteric redmouth disease · *Oncorhynchus mykiss* · Non-lethal method

Introduction

Since the past decade, there has been interest in the development of specific polymerase chain reaction (PCR) protocols, many of them based on the amplification of 16S rRNA genes, for detecting a variety of Gram-negative and Gram-positive bacterial fish pathogens (Beaz-Hidalgo et al. 2008; Osorio et al. 1999; Romalde et al. 2004). However, quantitative PCR (qPCR) technology is quickly replacing other molecular methods for detecting the nucleic acids of human and animal pathogens (Purcell 2011). The general principle of this method lies in their ability to detect and quantify DNA targets by monitoring PCR product accumulation during the early exponential phase of amplification, indicated by increased fluorescence. The advantage of qPCR assays is that it can be used to determine the pathogen presence and loads within fish tissues, which can be useful to assess for an ongoing carrier state or the onset of an epizootic by comparison with threshold limits of pathogen load prior to noticeable mortality (Taylor et al. 2009). Thus, real-time qPCR methods have been used for the detection of several microbial fish pathogens, such as *Lactococcus garvieae* (Jung et al. 2010), *Piscirickettsia salmonis* (Karatas et al. 2008), and *Aeromonas hydrophila* (Trakhna et al. 2009).

Yersinia ruckeri causes the enteric redmouth disease (ERM) primarily affecting salmonids. Diseased fish show typical symptoms such as dark skin, exophthalmia, and hemorrhaging of the eye and mouth (Busch 1978), but the bacterium may also persist in fish as an asymptomatic infection, until conditions of stress trigger disease outbreaks (Busch and Lingg 1975). In addition, *Y. ruckeri* can remain infective in the aquatic environment (Rodgers and Hudson

1985; Romalde et al. 1994), and it also has a biofilm-forming capacity (Coquet et al. 2002). Two biotypes have been described for this bacterial species. Biotype 1 (BT1) strains are positive for motility and lipase activity, whereas biotype 2 (BT2) strains are negative for both tests (Davies 1991). In addition, the species has been grouped into six serovars (Stevenson and Airdrie 1984) or four O-serotypes with different subgroups (Romalde et al. 1993) by using different serotyping systems. Motile serotype O1a *Y. ruckeri* strains have been associated to the most disease outbreaks (Davies 1991).

PCR-based DNA methods have been successfully used for detection of the fish pathogen *Y. ruckeri*, most of these PCR assays targeting the 16S rRNA gene (Gibello et al. 1999; Lejeune and Rurangirwa 2000). However, other candidate genes have offered improvements in conventional and qPCR assay specificity (Glenn et al. 2011; Keeling et al. 2011; Temprano et al. 2001), particularly to use on kidney tissues samples. These advances have allowed the fast and accurate detection of *Y. ruckeri* in fish samples but lack the ability to quantify the bacterial load in non-lethal samples.

In the current study, a qPCR method based on the *rec*A (DNA repair and recombination protein A) gene was performed and evaluated to develop a simple and robust approach for the detection and quantification of the pathogen *Y. ruckeri*. Furthermore, the optimized qPCR assay was used to analyze the distribution of this bacterium in several fish tissues, including blood samples, exposed to both motile and non-motile strains of *Y. ruckeri*.

Materials and methods

Bacterial strains

The bacterial strains used in this study are listed in Table 1. A total of 80 bacterial strains, including *Y. ruckeri* (*N*=45), *Yersinia* spp. (*N*=6), and unrelated bacterial species (*N*=29) were used to assess analytical specificity of the oligonucleotidic probe and primers. *Y. ruckeri* NCIMB 2194T^T (Hagerman strain) was used for the sensitivity assays and as positive control in every PCR and qPCR assay performed. *Y. ruckeri* strains CA2 (BT2/serotype O1a) isolated in Portugal in 2008, and PP31 (BT1/ serotype O1a) isolated in Spain in 1987, were used for the experimental challenges performed in rainbow trout for the detection and quantification of *Y ruckeri* in tissues. The bacteria were routinely grown on tryptone soy agar supplemented with 1 % NaCl (TSA-1) (Pronadisa, Spain), Columbia sheep blood agar (Oxoid Ltd., Spain), or Song medium (Song et al. 1988) at 15–22 °C for 24–48 h. Stock cultures were maintained frozen at −80 °C in tryptone soya broth supplemented with 1 % NaCl and 15 % glycerol.

Table 1 Bacterial strains used in this study

Bacterial species	PCR/qPCR detection
Yersinia ruckeri strains	
Type strain NCIMB 2194^T	+/+
Representative O-serotype strains[a]	
11.4 (O1a)	+/+
1531 (O1b)	+/+
RS2 (O2a)	+/+
11.29 (O2b)	+/+
RS6 (O2c)	+/+
11.47 (O3)	+/+
11.741/O1a	+/+
Isolated from outbreaks[b]	
Chile (*N*=10; biotypes, 1 and 2; serotypes, O1a, O1b, and O2b)	+/+
Portugal (*N*=5, included CA2 isolate; biotype, 2; serotype, O1a)	+/+
USA (*N*=11; biotypes, 1 and 2; serotypes, O1a and O1b)	+/+
Finland (*N*=2; biotype, 2; serotype, O1a)	+/+
UK (*N*=5; biotypes, 1 and 2; serotypes, O1a and O1b)	+/+
Peru (*N*=10; biotypes, 1 and 2; serotype O1a)	+/+
Spain (*N*=3, included PP31 isolate; biotype, 1; serotype, O1a and O2b)	+/+
Related species	
Yersinia enterolitica IP 383	−/−
Yersinia frederiksenii IP 16840	−/−
Yersinia intermedia IP 16835	−/−
Yersinia kristensenii IP 16832	−/−
Yersinia pestis 6/69 M	−/−
Yersinia pseudotuberculosis IP 2637	−/−
Unrelated species	
Alteromonas marina LMG 22057^T	−/−
Aeromonas hydrophila QSP 10.1	−/−
Aeromonas salmonicida ATCC 14174 ^{T}	−/−
Aerococcuss viridans CECT 978 ^{T}	−/−
Alivibrio fisheri NCIMB 1274	−/−
Alteromonas hispanica CECT 7067^T	−/−
Edwarsiella tarda NCIMB 2034	−/−
Enterobacter cloacae CECT 194^T	−/−
Escherichia coli ATCC 25992	−/−
Flavobacterium columnare PT4.1	−/−
Flavobacterium psycrophilum VTS4.1	−/−
Hafnia alvei VT 6.1	−/−
Lactococcus garvieae TW446B.3	−/−
Proteus vulgaris ATCC 13315^T	−/−
Pseudoalteromona carrageenovora NCIMB 302^T	−/−
Pseudoalteromona espejiana CECT 5002^T	−/−
Pseudoalteromona maricaloris LMG 19692	−/−
Pseusomona koreensis LMG 21318^T	−/−

Table 1 (continued)

Bacterial species	PCR/qPCR detection
Staphylococcus aureus ATCC 25923	−/−
Streptococcus parauberis RA99.1	−/−
Vibrio alginolyticus CCM 2578 ^{T}	−/−
Vibrio anguillarum ATCC 43306	−/−
Vibrio fluvialis ATCC33812	−/−
Vibrio harveyi CECT 525^{T}	−/−
Vibrio parahemolitycus ATCC 27969	−/−
Vibrio pelagius NCIMB 1900^{T}	−/−
Vibrio scopthalmi CECT 4638^{T}	−/−
Vibrio splendidus CECT 528^{T}	−/−
Vibrio vulnificus ATCC 27562^{T}	−/−

Strains IP 383, IP 2637, IP16832, IP 16835, IP 16840, and 6/69 M from E. Carniel (Institute Pasteur, France); QSP 10.1, PT4.1, VTS4.1, VT6.1, RA99.1, RA99.1, and TW446B.3 from laboratory collection

T type strain, *ATCC* American Culture Collection (USA), *NCIMB* National Collections of Industrial and Marine Bacteria (UK), *LMG* Laboratory for Microbiology (Ghent University, Belgium), *CECT* Spanish Collection of Type Cultures (Valencia, Spain.); strains IP 383, IP 2637, IP16832, IP 16835, IP 16840, and 6/69 M from E. Carniel (Institute Pasteur, France)

[a] Romalde et al. 1993

[b] *Y. ruckeri* isolates previously characterized by Bastardo et al. (2011a, b, 2012)

Design of *Y. ruckeri* primer/probe

The *rec*A gene partial sequence of *Y. ruckeri* ATCC 29473^{T} strain (GenBank accession FJ717382) was selected among other six housekeeping genes as a suitable target gene on basis of comparison with the closest relatives, *Yersinia* species, using the program BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi). *Y. ruckeri* species-specific nucleotide primers (YRA-F1, 5′-TCTGGACATCGCTCTGG-3′, and YRA-R2, 5′-AGTTTTTTTGCGTAGATAGGA-3′) and the TaqMan^{TM} probe consisted of an oligonucleotide (TATCGCCTCTGCACAGC) with a 5'-reporter fluorescent dye (FAM^{TM} 6-carboxyfluorescein) and a 3'-non-fluorescent quencher plus minor groove binder (MGB) were designed using DNAstar PrimerSelect program (Lasergene). Probe and primer specificity were assessed for possible similarity to sequences present in the GenBank database using BLAST.

Conventional PCR test and optimization of qPCR assay conditions

Primers were first tested against *Y. ruckeri* strains on a T Professional Basic thermocycler apparatus (Biometra, Goettingen, Germany). PCRs were carried out using the Ready-To-Go PCR beads kit (Pharmacia Biotech, Barcelona Spain) in a final volume of 25 μl containing 10 μM of each primer and 100 ng of template DNA. The PCR conditions were an initial denaturation of 95 °C for 3 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at temperature gradient from 52 to 62 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. Amplified PCR products were subjected to standard gel electrophoresis, stained with ethidium bromide and then illuminated by ultraviolet light; the expected size of the amplicons was confirmed by comparison with known DNA molecular weight markers (FastRuler^{TM} Low Range DNA ladder; Fermentas GMBH, Madrid, Spain). After determining the optimal annealing temperature, the detection limit using DNA purified from the *Y. ruckeri* NCIMB 2194^{T} strain was also determined.

Real-time PCR assays were conducted by utilizing the absolute quantification with the default thermocycling method on Mx3005P^{TM} Stratagene thermocycler (Agilent Technologies, Barcelona, Spain). All the amplifications were carried out in optical grade 96-well plates on a DNA Engine OPTICON^{TM} 2 system. Each reaction was run in triplicate in a master mix containing 12.5 μl of Maxima^{TM} Probe qPCR Master Mix (Fermentas), primers, and a probe with various concentrations of DNA samples as templates, to a final volume of 25 μl using nuclease-free water. The thermal cycling conditions were assessed testing different annealing temperatures (between 53 °C and 65 °C) during different times (15, 20, 30, 45 and 60 sec.) The optimal qPCR efficacy (100 %) was obtained using the follow protocol—1 cycle at 95 °C for 10 min followed by 40 cycles at 95 °C for 30 s, 62 °C for 1 min, and 72 °C for 1 min. Fluorescence intensity was expressed in delta reporters after background subtraction. The cycle threshold (C_T) value corresponded to the PCR cycle number at which an increase in reporter fluorescence above a baseline signals was first detected. The optimal of primers (Sigma-Aldrich, Madrid, Spain) and probe (Life Technologies SA, Madrid, Spain) were determined from different concentrations of primers (in 0.05, 0.2, 0.4, and 0.5 μM) and probe (in 0.05, 0.1, 0.15, and 0.20 μM) as the one which gave the highest recorded fluorescence and the lowest C_T. The qPCR amplicons were confirmed by electrophoresis in 2 % Seakem LE agarose gel and ethidium bromide staining.

Assessment of specificity and sensitivity of the qPCR

The specificity of qPCR assay was evaluated by amplification of DNA templates from all the *Y. ruckeri* strains and the representatives of the other bacterial species (listed in Table 1) under conventional and real-time PCR conditions optimized as was specified above. For sensitivity testing, DNA obtained from a *Y. ruckeri* NCIMB 2194^{T} culture at 10^{9} colony-forming units (CFU)ml^{-1} was serially diluted tenfold and prepared as template for real-time PCR. In

addition, the CFU ml^{-1} in the original bacterial suspension were also determined by the viable cell plate count method, to calculate the sensitivity of the qPCR assay in bacterial cell numbers. The detection limit was determined as the lowest concentration (within the linear range) that produced an amplification signal. The DNA concentration was converted to genomics equivalents based on the assumption that single copies of the target sequences was present in the genome. A lineal regression line analysis was performed, and the coefficient of determination (R^2) was calculated.

The analytical sensitivity was also determined for DNA from seeded fish tissues to assess the effect of any PCR inhibitors present in the tissues. Portions of individualized organs including liver, kidney, spleen, and blood were obtained from healthy rainbow trout and divided into 50-mg pieces or 100-μl (in case of blood samples) aliquots and then homogenized using a Stomacher Lab-Blender 80 (Lab System Fisher Scientific, Pittsburgh, Pennsylvania, USA) for 1 min. Next, tissues were separately seeded with 10 μl of different dilutions (10^9 to 10 UFC ml^{-1}) of a culture suspension of the *Y. ruckeri* NCIMB 2194^T in phosphate-buffered saline (pH 7.4). After incubation for 1 h at room temperature, tissues lyses and total DNA purification were performed using the Easy-DNA™ kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions for small amounts of cells, tissues, and blood. Unseeded tissues employed as negative controls were processed in the same manner. For real-time PCR, 5 and 8 μl of the extracted DNA solutions using the different organs and blood were used, respectively. The detection limits for each tissue employed were determined as was described earlier, and the lowest dilution showing amplification was then correlated with the number of bacterial cells that were added in tissues (CFU per gram) and blood (CFU/100 μl^{-1}). Sensitive experiments were performed three times using five replicates for DNA dilution used and three replicates for each DNA extracted from seeded tissues.

Reproducibility of the qPCR assay

The intra- and inter-run percentages of coefficient of variation (% CV) were established to assess the reproducibility of the real-time PCR assay. For the intra-run % CV, the test was developed using nine concentrations (tenfold dilutions) of *Y. ruckeri* NCIMB 2194^T with five replicates for each concentration. The inter-run % CV experiment was conducted three times for both pure culture DNA and DNA extracted from seeded tissues. Individual assays were performed on seven concentrations (tenfold dilutions) of DNA and different concentrations (from 10^9 to 10^3 UFC ml^{-1}) of *Y. ruckeri* NCIMB 2194^T seeded into each liver, kidney, and spleen and blood. The % CV was determined for each of the concentrations of DNA or seeded tissues.

Experimental infections

Two challenges were performed to test the infectivity of two *Y. ruckeri* (CA2 and PP31) strains in rainbow trout (*Onchorhynchus mykiss*) obtained from an ERM-free farm in Galicia (NW, Spain). Each challenge was performed separately and consisted of groups of five fish (8 g average body weight), intraperitoneally injected with 0.1 ml of different tenfold dilutions of the bacteria ranging from 1.5×10^8 to 15 CFU ml^{-1} for PP31 isolate and from 1.3×10^8 to 13 CFU ml^{-1} for CA2 isolate. Fish used as negative controls received 0.1 ml of saline solution. Fish were maintained in 50 l aquaria at 14±1 °C with aeration, and mortalities were daily scored for a period of 15 days.

Biopsied organs (liver, kidney, and spleen) were removed from dead fish in each experimental group and aliquoted separately in 50-mg portions. Blood samples were pooled for each group and were obtained from caudal vein of the fish by cutting off the tail and using a Pasteur pipette. Samples were stored at −20 °C until DNA extraction could be performed. In addition, all dead fish were examined to confirm the re-isolation of the inoculated strains from the internal organs (liver, kidney, and spleen) by streaking directly onto TSA-1 plates. Mortalities were considered to be due to *Y. ruckeri* if the challenged strains were isolated as pure culture from internal organs and identified by slide agglutination with a specific anti-*Y. ruckeri* O1 serum, obtained from our laboratory as previously described (Bastardo et al. 2011a). After a 15-day period, survivor fish were sacrificed by anesthetic overdose with tricaine (1 gl^{-1}; Sigma, Madrid, Spain) and bacteriologically analyzed, and tissues were treated in the same manner as those from dead fish. The Ethics Committee for Animal Experiments of the University of Santiago de Compostela approved the protocols of this study.

Total DNA extractions from all tissues and qPCR for the detection and quantification of *Y. ruckeri* were performed as described earlier. The C_T of samples obtained by qPCR was used to calculate the total number of CFU^{-1} and CFU/100 μl^{-1}, present in tissues (liver, kidney, and spleen) and blood sample, respectively, on bases on the sensitivity results.

Statistical analysis

Linear regression of the cycle threshold values versus seeded cells numbers (enumerated by plate count method) was conducted for all the sensitivity assays performed. ANOVA analysis was performed to compare the results of the quantified DNA bacteria obtained among the different dilutions in the challenges. The level of significance for all tests was assessed at 0.05. Chi-squared analysis was performed to determine differences in the C_T values determined between

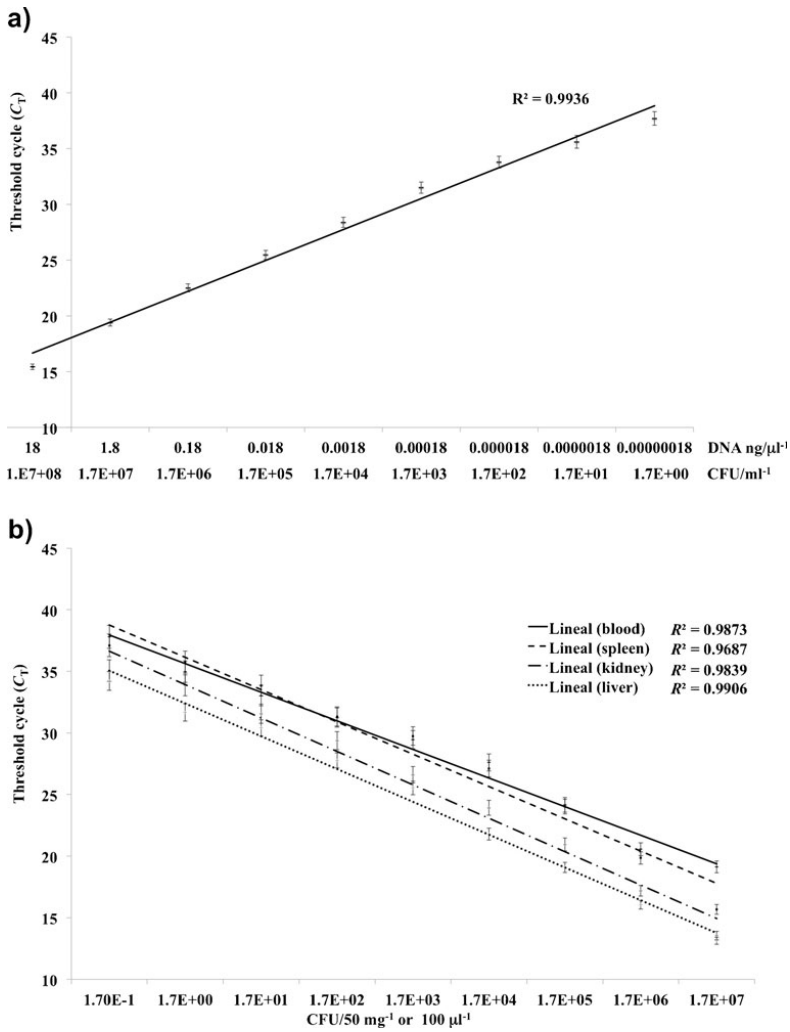

Fig. 1 Standard curves and lineal regression analysis obtained by real-time PCR assay from: **a** tenfold serial dilutions of *Y. ruckeri*, and **b** *Y. ruckeri* DNA extracted from inoculated liver, kidney, spleen tissues (CFU g^{-1}) and blood (CFU 100 μl^{-1}). Error *bars* represent n=5±SD (standard deviation) in **a**, and N=3±SD in **b**

both *Y. ruckeri* (PP31 and CA2) strains used in the challenge experiments. All statistical analyses were performed using the statistical package SPSS for Windows version 11.5.

Results

Specificity of PCR reaction primers

After conventional PCR amplification, a unique of 188 bp band amplified with the *rec*A YR01 and the *rec*A YR02 primer set was detected at optimal annealing temperature of 62 °C. The expected PCR product of 188 bp was common to all *Y. ruckeri* strains (data not shown). The detectable minimum concentration was determined to be 1 pg of DNA. No amplification of PCR products from any other non-target bacterial DNA belonging related species was observed with the primers used, neither for the other fish pathogen tested (Table 1).

Real-time PCR assay and analytical specificity and sensitivity

Following the optimization of qPCR assay, primers and oligonucleotidic probe were used at the optimal concentration of 0.2 of and 0.15 μM, respectively. The optimal 100 %

of qPCR efficacy was only obtained using an annealing temperature of 62º C during 1 min per cycle. The analytical specificity was confirmed to be 100 % through analysis of related species and other fish bacterial pathogens (Table 1). The real-time PCR assay amplified the DNA of all the *Y. ruckeri* strains tested. Amplified PCR product size was verified by gel electrophoresis, visualizing a 188-bp-long amplicon. Positive signals were detected in all dilutions from purified *Y. ruckeri* genomic DNA. The minimum level of detection obtained from pure culture was 1.8 ag μl qPCR mixture^{-1}, or the equivalent to 1.7 CFU ml^{-1}. High correlation between the numbers of *Y. ruckeri* cells and C_T values was detected with a R^2=0.994 and PCR efficacy of 99 % (Fig. 1a).

The C_T values obtained from the analysis of tissue samples were extrapolated to the corresponding previously calculated standard curves. The quantification curves obtained for the different tissues analyzed were similar exhibiting correlations (R^2 values) between 0.9687 and 0.9906 (Fig. 1b). The detection limits achieved in the sensitivity assays for seeded tissues (liver, kidney, and spleen) and blood were 3.4 CFU g^{-1} and 0.34 CFU/100 μl^{-1}, respectively. These demonstrated that the presence of fish tissue did not affect the qPCR detection of *Y. ruckeri* NCIMB 2194^T. No statistical differences were determined in the C_T values among the different tissues (Fs=0.79; p>0.05). No amplification was observed in qPCR assay performed using DNA purified from non-seeded kidney, liver, spleen tissues, and blood or negative control. Additional standard curves made in parallel with some of the samples rendered similar values of CFU, confirming the suitability of this method for quantitative and reproducible measurement of CFU loads (data not shown).

Reproducibility of the qPCR assay

The CV percentages for intra- and inter-run tests performed using genomic DNA templates as well as bacterial DNA extracted from seeded tissues showed high degree of repeatability and reproducibility for the qPCR assay (Tables 2 and 3). The intra- and inter-run % CVs for purified DNA ranged from 0.42 to 2.94 and from 0.88 to 6.34, respectively. For seeded tissues, the intra-run % CV was found between 0.07 and 9.48.

Experimental infections

Both isolates caused high mortalities in fish. Higher mortality value was observed in fish challenged using the non-motile CA2 *Y. ruckeri* strain with 100 % of cumulative mortality observed using dilutions between 1.3×10^8 and 1.3×10^3 UFC/ml (Fig. 2a). In fish challenged using the motile PP31 *Y. ruckeri* isolate, the highest cumulative mortality was observed when dilutions between 1.5×10^8 and 1.5×10^4 UFC/ml were used (Fig. 2b). C_T values obtained in blood and kidney fish tissues, assessed using both PP31 and CA2 isolates, were higher than those detected from survivals fish. Similar results were observed in liver and spleen tissues (data not shown). Table 4 shows the results obtained of the quantification of PP31 and CA2 *Y. ruckeri* cells in the different fish tissues, at several dilutions inoculated in each challenge performed. The mean bacterial loads for strain PP31 did not differ from that of strain CA2, regardless the different tissues analyzed (p>0.05). In dead fish tissues, the bacterial load ranged from 1.3×10^7 (at C_T values 22.4±1.1) to 9.8×10^{10} (at C_T values 22.7±7.0)CFU g^{-1} for liver, kidney, and spleen tissues and from 7.5×10^7 (at C_T values 22.7±0.2) to 9.5×10^9 (at C_T values 18.3±0.7)CFU/100 μl^{-1} in blood samples. These values were detected in tissues from the dead fish inoculated with doses between 10^7 and 10^4 CFU/ml^{-1} for the motile PP31 strain and doses from 10^7 to 10^2 CFU/ml^{-1} in the case of the non-motile CA2 isolate (Table 4; Fig. 2). Although no significant differences

Table 2 Percentage coefficient of variation (% CV) for inter- and intra-run repeatability of real-time PCR assay for genomic DNA templates

DNA (ng/μl^{-1})	% CV intra-run	% CV inter-run
180	2.94	6.34
18	1.73	6.07
1.8	1.16	1.28
0.18	0.42	0.91
0.018	2.31	1.84
0.0018	2.92	0.88
0.00018	1.15	3.38
0.000018	2.52	5.18
0.0000018	1.41	3.08

Results are for five replicates for each concentration of DNA used

Table 3 Percentage coefficient of variation (% CV) for inter-run repeatability of real-time PCR assay for bacterial DNA purified from inoculated tissues

	% CV inter-assay			
CFU g^{-1}	Liver	Kidney	Spleen	Blood
10^7	1.62	2.45	9.48	1.48
10^6	3.75	1.65	4.42	2.71
10^5	6.15	3.31	2.94	5.48
10^4	4.41	1.21	0.96	4.56
10^3	4.35	3.45	2.76	0.47
10^2	6.24	2.79	3.88	2.09
10^1	5.09	0.07	0.67	1.54

Results are in triplicate for each bacterial dilution seeded in each tissue

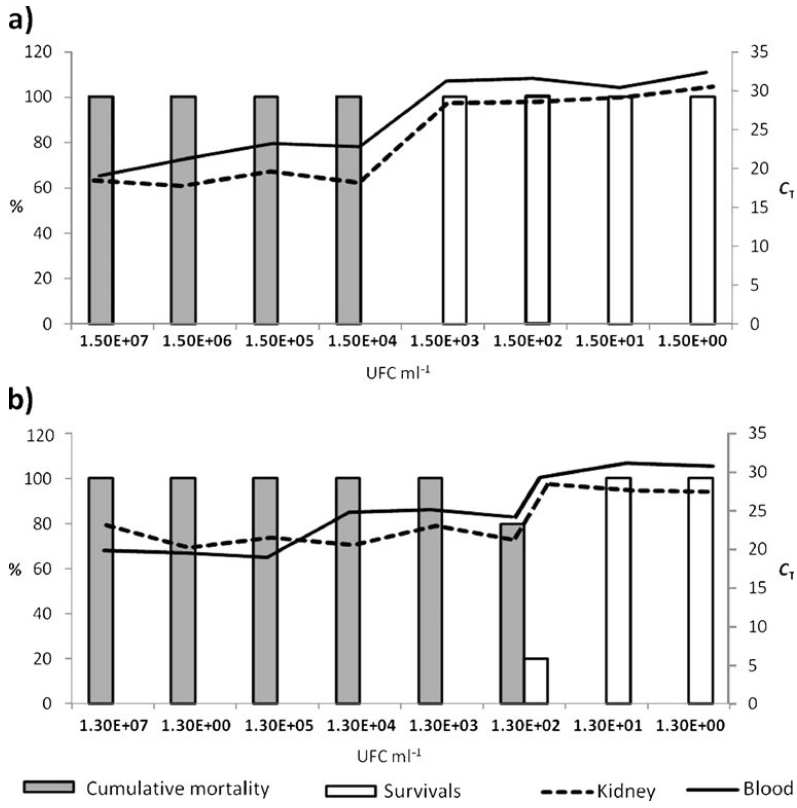

Fig. 2 Mortality cumulative, survival percentages, and C_T values obtained in kidney and blood samples from fish inoculated with different concentrations of *Y. ruckeri*: **a** PP31 isolate and **b** CA2 isolate

were detected, C_T and/or bacterial load (CFU) values were generally higher in blood.

Conversely, the bacterial loads determined in the different tissues of surviving fish were lower, ranging from 8.8×10^2 (C_T=33.2±0.2) to 7.2×10^5 (C_T=27.4±2.4) in liver, kidney, and spleen tissues and between 5.1×10^3 (C_T=32.4±0.0) and 6.2×10^4 (C_T=28.9±0.2)CFU/100 μl^{-1} in blood. Furthermore, significant differences ($p<0.05$) were observed in the bacterial load between dead fish and survivors. Plating recovery of the inoculated strains was obtained from tissues from fish that died during the 15-day period post-infection. However, in surviving fish inoculated with less than 10^2 CFU ml^{-1}, detection of *Y. ruckeri* was only possible by means of the qPCR assay.

Discussion

Clinical laboratories are increasing the use of PCR procedures to complement or replace classic diagnostic assays, often in the context of prevention programs or to ensure the identification of a pathogen (Cunningham 2002). The occurrence of recent outbreaks caused by *Y. ruckeri* in vaccinated fish has evidenced an urgent need to develop sensitive and reliable methods for fast detection of this pathogen, not only in clinically infected animals but also in carrier fish.

In this study, we developed a rapid and specific procedure for detection and quantification of *Y. ruckeri* by conventional PCR and qPCR. Under the optimized conditions, qPCR results can be obtained approximately 2 h following to DNA extraction. Other studies have reported shorter cycling conditions, specifically in the annealing step (Glenn et al. 2011; Keeling et al. 2011). The conditions required in this study may probably be related to requirements of the polymerase employed (Suzuki and Sakai 2007). A broad range of bacterial cell numbers (from 1 up to 10^8 CFU) can be measured using qPCR (Nadkarni et al. 2002). In this study, the assay demonstrated to be highly sensitive with a minimum level of DNA detection of 1.8 ag, corresponding to approximately two *Y. ruckeri* cells (CFU per milliliter). This limit of detection was lower than those achieved in other studies for fish pathogens (Balcázar et al. 2007; Jung et al. 2010; Keeling et al. 2011; Suzuki and

Table 4 Quantification of *Y. ruckeri* on fish tissues challenged with both CA2 and PP31 strains

	Liver		Kidney		Spleen		Blood	
Dose	CA2	PP31	CA2	PP31	CA2	PP31	CA2	PP31
Dead fish								
10^7	6.79×10^7 (21.48±2.10)	7.90×10^7 (20.90±1.61)	1.20×10^8 (23.10±3.08)	1.04×10^9 (18.45±0.35)	1.59×10^9 (21.89±2.96)	1.89×10^8 (24.71±3.38)	1.06×10^9 (20.04±0.26)	9.53×10^9 (18.30±0.71)
10^6	2.66×10^7 (21.68±3.91)	3.80×10^7 (23.27±1.06)	2.83×10^8 (20.23±1.11)	6.64×10^9 (17.79±3.42)	8.29×10^7 (24.83±2.31)	1.45×10^8 (24.40±1.74)	2.37×10^9 (19.75±1.63)	8.61×10^9 (20.61±1.33)
10^5	2.45×10^8 (20.93±1.73)	1.75×10^9 (21.89±6.16)	8.62×10^7 (21.51±0.77)	7.53×10^9 (19.60±4.20)	4.42×10^7 (25.33±1.94)	9.05×10^9 (23.44±4.90)	2.46×10^9 (19.18±0.25)	7.55×10^7 (22.69±0.16
10^4	1.28×10^7 (22.39±1.08)	1.03×10^8 (21.11±2.23)	3.37×10^8 (20.56±3.21)	1×10^9 (18.19±1.36)	2.46×10^8 (25.50±4.19)	1.92×10^9 (20.80±2.13)	3.41×10^7 (24.93±6.10)	8.61×10^8 (22.22±3.80)
10^3	3.74×10^8 (21.11±3.49)	ND	2.13×10^8 (23.03±3.54)	ND	9.81×10^{10} (22.67±7.00)	ND	4.06×10^7 (25.26±3.74)	ND
10^2	9.44×10^7 (22.89±3.44)	ND	2.07×10^8 (21.12±1.86)	ND	1.11×10^8 (24.10±1.32)	ND	4.21×10^7 (24.35±2.47)	ND
10^1	ND	ND	ND	ND	ND	ND	ND	ND
Survival fish								
10^3	NS	2.91×10^5 (29.77±2.70)	NS	3.90×10^5 (28.40±1.98)	NS	6.32×10^5 (28.99±0.01)	NS	1.12×10^4 (31.20±0.57)
10^2	7.86×10^4 (28±0.70)	2.13×10^5 (29.36±4.32)	1.94×10^5 (28.4±1.98)	1.09×10^5 (28.56±2.34)	1.53×10^5 (31.1±0.14)	2.0×10^5 (29.8±0.57)	6.23×10^4 (28.95±0.21)	3.02×10^4 (31.55±1.69)
10^1	9.93×10^4 (28.47±1.79)	1.07×10^5 (28.41±1.83)	6.20×10^5 (27.61±1.54)	1.16×10^5 (29.14±0.22)	2.85×10^5 (30.79±0.15)	No C_T	1.44×10^4 (31.20±0.14)	2.97×10^4 (30.3 ±0.21)
10^0	2.60×10^4 (29.50 ±0.71)	8.88×10^2 (33.22±0.16)	7.18×10^5 (27.45±2.40)	2.83×10^4 (30.50±0.35)	No C_T	No C_T	3.49×10^4 (30.74±1.44)	5.10×10^3 (32.40±0.03)

Values shown represent the averages of CFU obtained by triplicate in qPCR reaction. In parentheses, mean of cycle threshold and deviation standard values correspondent

ND no dead fish, *NS* no survivor fish; *No C*T no cycle threshold value obtained

Sakai 2007). The primers/probe set was 100 % specific for *Y. ruckeri rec*A gene sequence, which indicates that the likelihood of false-positives being generated by contaminating species of bacteria is extremely low. The assay detected all the biotypes and serotypes of *Y. ruckeri* included in the assessment, demonstrating that this qPCR assay can be potentially suited to use in those areas where BT2 and other serotypes have been reported as an increasing problem (Bastardo et al. 2012; Welch et al. 2011). The good specificity and high sensitivity (although slightly lower than in qPCR) obtained by conventional PCR using the primers YRA-F1 and YRA-R2 constitute an additional value to this study, since it can be relevant for many diagnostic laboratories that still cannot have a real-time machine.

Bacterial culture from organs obtained during necropsy is necessary for the diagnosis of ERM, because the clinical signs of this disease are variable and not distinctive (Horne and Barnes 1999). At the moment, kidney tissue has proved to be the best source of pathogen for culture and PCR analysis. Liver, spleen, and blood have proved less satisfactory for PCR analysis (Argenton et al. 1996; Balcázar et al. 2007). The R^2 obtained in this assay for purified DNA and seeded liver, kidney, and spleen tissues, as well as for blood, showed a high linear correlation with template concentrations. Moreover, the analytical sensitivity obtained in this study for seeded tissues (kidney, liver, and spleen) can be comparable to those obtained in other studies (Balcázar et al. 2007; Suzuki and Sakai 2007). However, this study showed increased sensitivity in trout kidney tissue in comparison to previous works using PCR and qPCR assays which reported 2×10^4 and 3×10^3 CFU g^{-1}, respectively (Gibello et al. 1999; Keeling et al. 2011). As could be expected, quite high variability in the standard deviation (SD) values was observed in some cases, which is probably related with the variable response to the pathogen and/or the behavior of the pathogen within the host, which can to vary from fish to fish.

The bacterial loads detected in this study for fish tissues were greater in blood compared with liver, kidney, and spleen, and especially in tissues from dead fish infected with the highest number of *Y. ruckeri* cells (>1×10^5 CFU ml^{-1}). Conversely, no differences in the bacterial loads were observed among the different tissues analyzed from the survival fish, which can be indicative of a generalized

septicemia. Blood testing would be preferable to analyses based on kidney, liver, or spleen tissues because it does not require the fish death, being therefore suitable for frequent in situ health monitoring on fish farms. Previously, problems were reported in the detection of *Y. ruckeri* and other fish pathogens in blood samples using PCR procedures (Argenton et al. 1996; Klein et al. 1997), which could be explained by PCR inhibitors and/or failure of the extraction methods. In the present study, the detection and quantification of *Y. ruckeri* in blood samples using the qPCR protocol proved to be successful, detecting down to 0.34 CFU 100 μl^{-1}. Other studies have also reported acceptable levels of detection for *Y. ruckeri* (Altinok et al. 2001) and other fish pathogenic bacteria in blood samples using PCR assays (Bilodeau and Waldbieser 2003).

Although *Y. ruckeri* appears to be predominantly an extracellular pathogen, a high proportion of bacteria could reside inside granular cells earlier in infection or in the chronic form of the disease (Welch and Wiens 2005). The percentage of these granular cell populations was reported to be highest in the peripheral blood and lowest in the anterior kidney, consistent with known properties of rainbow trout phagocytic cells (Köllner et al. 2001; Moritomo et al. 2003). Thus, the data obtained in this study are consistent with migration of granulocytes from the anterior kidney to the periphery during *Y. ruckeri* infection (Welch and Wiens 2005).

In the current study, presence of *Y. ruckeri* was not always directly related to fish mortality. Mortality did not occur until the bacterial loads in the tissues exceeded 10^6 CFU g^{-1}. This finding shows the usefulness of the qPCR assay to detect variations of *Y. ruckeri* load within fish tissues and can indicate that this level of bacterial load can be a critical threshold between an epizootic and non-epizootic or carrier state. The detection of these asymptomatic carriers is very important in order to prevent the transmission and diffusion of ERM in farms (Busch and Lingg 1975).

Differences in bacterial loads of *Y ruckeri* were not observed among tissues of fish inoculated with both motile (PP31) and non-motile (CA2) isolates used in this assessment. However, different percentages of mortality were determined. This finding may indicate that, while the pathogen is widespread, different strains can show different degrees of virulence. At present, the ultimate cause of the increase in virulence of *Y. ruckeri* BT2 isolate is unclear. One possible mechanism, which also helps to explain the emergence of non-motile *Y. ruckeri*, is the mutational loss of components necessary for flagellar secretion (Evenhuis et al. 2009). However, there is no evidence that avoidance of the trout anti-flagellin inflammatory response is the mechanism driving the evolution of the BT2 phenotype. Conversely, a robust response even in the absence of flagellin expression has been observed (Wiens and Vallejo 2010).

In summary, the qPCR assay developed in this study is successful for the detection and quantitative measurement of *Y. ruckeri*, not only from pure cultures of the pathogen but also from fish tissue homogenates and blood. Therefore, this qPCR assay offers a useful alternative to the microbiological approach for the rapid, specific, and non-lethal diagnosis of *Y. ruckeri* in infected fish, as well as for potential detection of asymptomatic or carrier populations in fish farms.

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References

Altinok I, Grizzle JM, Liu Z (2001) Detection of *Yersinia ruckeri* in rainbow trout blood by use of the polymerase chain reaction. Dis Aquat Org 44:29–34

Argenton F, De Mas S, Malocco C, Dalla Valle L, Giorgetti G, Colombo L (1996) Use of random DNA amplification to generate specific molecular probes for hybridization tests and PCR-based diagnosis of *Yersinia ruckeri*. Dis Aquat Org 24:121–127

Balcázar JL, Vendrell D, de Blas I, Ruiz-Zarzuela I, Gironés O, Múzquiz JL (2007) Quantitative detection of *Aeromonas salmonicida* in fish tissue by real-time PCR using self-quenched, fluorogenic primers. J Med Microbiol 56:323–328

Bastardo A, Bohle H, Ravelo C, Toranzo AE, Romalde JL (2011a) Serological and molecular heterogeneity among *Yersinia ruckeri* strains isolated from farmed Atlantic salmon *Salmo salar* in Chile. Dis Aquat Org 93:207–214

Bastardo A, Sierralta V, León J, Ravelo C, Romalde JL (2011b) Phenotypical and genetic characterization of *Yersinia ruckeri* strains isolates from recent outbreaks in farmed rainbow trout *Onchorhyncus mykiss* (Walbaum) in Peru. Aquaculture 317:229–232

Bastardo A, Ravelo C, Romalde JL (2012) A polyphasic approach to study the intraspecific diversity of *Yersinia ruckeri* strains isolated from recent outbreaks in salmonid culture. Vet Microbiol. doi:10.1016/j.vetmic.2012.05.024

Beaz-Hidalgo R, Magi GE, Balboa S, Barja JL, Romalde JL (2008) Development of a PCR protocol for the detection of *Aeromonas salmonicida* in fish by amplification of the *fst*A (ferric siderophore receptor) gene. Vet Microbiol 128:386–394

Bilodeau AL, Waldbieser GC, Terhune JS, Wise DJ, Wolters WR (2003) A Real-time polymerase chain reaction assay of the bacterium *Edwardsiella ictaluri* in channel catfish. J Aquat Anim Health 15:80–86

Busch RA (1978) Enteric redmouth disease (Hagerman strain). Mar Fish Rev 40:42–51

Busch RA, Lingg AJ (1975) Establishment of an asymptomatic carrier state infection of enteric redmouth disease in rainbow trout (*Salmo gairdneri*). J Fish Res Board Can 32:2429–2432

Coquet L, Cosette P, Quillet L, Petit F, Junter GA, Jouenne T (2002) Occurrence and phenotypic characterization of *Yersinia ruckeri* strains with biofilm-forming capacity in a rainbow trout farm. Appl Environ Microbiol 68:470–475

Cunningham CO (2002) Molecular diagnosis of fish and shellfish diseases: present status and potential use in disease control. Aquaculture 206:19–55

Davies RL (1991) Clonal analysis of *Yersinia ruckeri* based on biotypes, serotypes and outer membrane protein types. J Fish Dis 14:221–228

Evenhuis JP, Lapatra SE, Verner-Jeffreys DW, Dalsgaard I, Welch TJ (2009) Identification of flagellar motility genes in *Yersinia ruckeri* by transposon mutagenesis. Appl Environ Microbiol 75:6630–6633

Gibello A, Blanco MM, Moreno AM, Cutuli MT, Domenech A, Domínguez I, Fernández-Garayzábal JF (1999) Development of a PCR assay for detection of *Yersinia ruckeri* in tissues of inoculated and naturally infected trout. Appl Environ Microbiol 65:346–350

Glenn R, Taylor PW, Hanson KC (2011) The use of real-time PCR primer/probe set to observe infectivity of *Yersinia ruckeri* in Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), and steelhead trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis 34:783–791

Horne MT, Barnes AC (1999) Enteric redmouth disease (*Yersinia ruckeri*). In: Woo PTK, Bruno DW (eds) Fish diseases and disorders, vol. 3: viral, bacterial and fungal infections. CABI Publishing, Oxford, pp 455–477

Jung MY, Chang Y-H, Kim W (2010) A real-time PCR assay for detection and quantification of *Lactococcus garvieae*. J Appl Microbiol 108:1694–1701

Karatas S, Mikalsen J, Steinum TM, Taksdal T, Bordevik M, Colquhoun DJ (2008) Real time PCR detection of *Piscirickettsia salmonis* from formalin-fixed paraffin-embedded tissues. J Fish Dis 31:747–753

Keeling SE, Johnston C, Wallis R, Brosnahan CL, Gudkovs N, McDonald WL (2011) Development and validation of real-time PCR for the detection of *Yersinia ruckeri*. J Fish Dis 35:119–125

Klein A, Barsuk R, Dagan S, Nusbaum O, Shouval D, Galun E (1997) Comparison of methods for extraction of nucleic acid from hemolytic serum for PCR amplification of hepatitis B virus DNA sequences. J Clin Microbiol 35:1897–1899

Köllner B, Blohm U, Kotterba G, Fischer U (2001) A monoclonal antibody recognising a surface marker on rainbow trout (*Oncorhynchus mykiss*) monocytes. Fish Shellfish Immunol 11:127–142

Lejeune JR, Rurangirwa FR (2000) Polymerase chain reaction for definitive identification of *Yersinia ruckeri*. J Vet Diagn Invest 12:558–561

Moritomo T, Serata K, Teshirogi K, Aikawa H, Inoue Y, Itou T, Nakanishi T (2003) Flow cytometric analysis of the neutrophil respiratory burst of ayu, *Plecoglossus altivelis*: comparison with other fresh water fish. Fish Shellfish Immunol 15:29–38

Nadkarni MA, Martin FE, Jacques NA, Hunter N (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 148:257–266

Osorio CR, Collins MD, Toranzo AE, Barja JL, Romalde JL (1999) 16 S rRNA gene sequence analysis of *Photobacterium damselae* and nested PCR method for rapid detection of the causative agent of fish pasteurellosis. Appl Environ Microbiol 65:2942–2946

Purcell MH, Getchell RG, McClure CA, Gerver KA (2011) Quantitative polymerase chain reaction (PCR) for detection of aquatic animal pathogens in a diagnostic laboratory setting. J Aquat Anim Health 23:148–161

Rodgers CJ, Hudson EB (1985) A comparison of two methods for isolation of *Yersinia ruckeri* from rainbow trout (*Salmo gairdneri*). Bull Eur Ass Fish Pathol 5:92–93

Romalde JL, Margariños B, Barja JL, Toranzo AE (1993) Antigenic and molecular characterization of *Yersinia ruckeri*. Proposal for a new intraspecies classification. Syst Appl Microbiol 16:411–419

Romalde JL, Barja JL, Magariños B, Toranzo AE (1994) Starvation-survival processes of the bacterial fish pathogen *Yersinia ruckeri*. Syst Appl Microbiol 17:161–168

Romalde JL, López-Romalde S, Ravelo C, Magariños B, Toranzo A (2004) Development and validation of a PCR-based protocol for the detection of *Pseudomonas anguilliseptica*. Fish Pathol 39:33–41

Song YL, Fryer JL, Rohovec JS (1988) Comparison of six media for the cultivation of *Flexibacter columnaris*. Fish Pathol 23:91–94

Stevenson RMW, Airdrie DW (1984) Serological variation among *Yersinia ruckeri* strains. J Fish Dis 7:247–254

Suzuki K, Sakai DK (2007) Real-time PCR for quantification of viable *Renibacterium salmoninarum* in chum salmon. Dis Aquat Org 74:209–223

Taylor PW, Glenn RA, Gutenberger SK, Sorenson R (2009) Development and refinement of PCR and qPCR assays for the rapid detection of *Renibacterium salmoniarum*, the causative agent of bacterial kidney disease, in Chinook salmon at the Warm Springs National Fish Hatchery. U. S. Fish and Wildlife Service, Abernathy Fish Technology Center, Longview, WA

Temprano A, Yugueros J, Hernanz C, Sánchez M, Berzal B, Luengo JM, Naharro G (2001) Rapid identification of *Yersinia ruckeri* by PCR amplification of *yru*I-*yru*R quorum sensing. J Fish Dis 24:253–261

Trakhna F, Harf-Monteil C, Abdel Nour A, Maaroufi A, Gadonna-Widehem P (2009) Rapid *Aeromonas hydrophila* identification by TaqMan PCR assay: comparison with a phenotypic method. Lett Appl Microbiol 49:186–190

Welch TJ, Wiens GD (2005) Construction of a virulent, green fluorescent protein tagged *Yersinia ruckeri* and detection in trout tissues after intraperitoneal and immersion challenge. Dis Aquat Org 67:267–272

Welch TJ, Verner-Jeffreys DW, Dalsgaard I, Wiklund T, Evenhuis JP, Cabrera JA, Hinshaw JM, Drennan JD, LaPatra SE (2011) Independent emergence of *Yersinia ruckeri* biotype 2 in the United States and Europe. Appl Environ Microbiol 77:3493–3499

Wiens GD, Vallejo RL (2010) Temporal and pathogen-load dependent changes in rainbow trout (*Oncorhynchus mykiss*) immune response traits following challenge with biotype 2 *Yersinia ruckeri*. Fish Shellfish Immunol 29:639–647

FIGURAS COMPLEMENTARIAS CAPÍTULO IV.

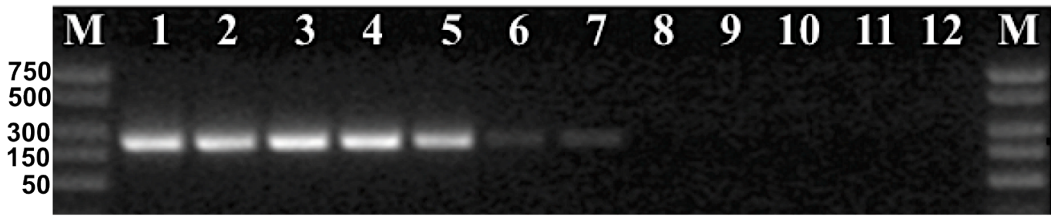

Figura complementaria 1. Sensibilidad de detección de la PCR convencional de *Yersinia ruckeri* utilizando los cebadores YRA-F1 y YRA-F2. Patrón electroforético obtenido de las amplificaciones (188 pb) de las siguientes diluciones (líneas) en gel de agarosa al 2%: M, marcador de peso molecular (750–50 pares de bases); Líneas: 1, 1000; ng; 2, 100 ng; 3, 10 ng; 4, 1ng; 5, 100 pg; 6, 10 pg; 7:,1 pg.

Figura complementaria 2. Especificidad de los cebadores YRA-F1 y YRA-F2. Productos de las amplificaciones (188 pb) en PCR convencional de DNAs extraídos de diferentes especies bacterianas. **A**) M: marcador de peso molecular (2000-150 pb), Líneas: 1, control negativo; 2, *Yersinia enterolitica* IP 383; 3: *Y. frederiksenii* IP 16840; 4: *Y. intermedia* IP 16835; 5: *Y. kristensenii* IP 16832; 6: *Y. pestis* 6/69M; 7: *Y. Pseudotuberculosis*; 8: *Alteromonas marina* LMG 22057^{T}; 9: *Aeromonas hydrophila* QSP 10.1; 10: *A. salmonicida* ATCC 14174 ^{T}; 11: *Aerococcuss viridans* CECT 978 ^{T}; 12: *Allivibrio fisheri* NCIMB 1274; 13: *Al. hispanica* CECT 7067^{T}; 14: *Edwarsiella tarda* NCIMB 2034; 15: *Enterobacter cloacae* CECT 194^{T}; 16: *Escherichia coli* ATCC 25992; 17: *Flavobacterium columnare* PT4.1; 18: *F. psycrophilum* VTS4.1; 19: *Hafnia alvei* VT 6.1; 20: *Y. ruckeri* NCIMB 2194^{T}; 21: *Lactococcus garvieae* TW446B.3; 22: *Proteus vulgaris* ATCC 13315^{T}; 23: *Pseudoalteromonas carrageenovora* NCIMB 302^{T}. 24: control negativo. **B**) M: marcador de peso molecular (pb), Líneas: 1: *P. espejiana* CECT 5002^{T}; 2: *Y. ruckeri* NCIMB 2194^{T}; 3: *P. maricaloris* LMG 19692; 4: *Pseudomonas koreensis* LMG 21318^{T}; 5: *Staphylococcus aureus* ATCC 25923; 6: *Streptococcus parauberis* RA99.1; 7: *Vibrio alginolyticus* CCM 2578 ^{T}; 8: *V. vulnificus* ATCC 27562^{T}; 9: *V. anguillarum* ATCC 43306; 10: *V. fluvialis* ATCC33812; 11: *V. harveyi* CECT 525^{T}; 12: *V. parahemolitycus* ATCC 27969; 13: *V. pelagius* NCIMB 1900^{T}; 14: *V. scopthalmi* CECT 4638^{T}; 15: *V. splendidus* CECT 528^{T}; 16; *V. tasmaniensis* LMG 21574^{T}; 17: control negativo.

Figura complementaria 3. Productos de las amplificaciones (188 pb) en PCR convencional de DNAs extraídos de diferentes cepas de *Yersinia ruckeri* **A**) M: marcador de peso molecular (750-100 pb); Línea 1: *Y. ruckeri* NCIMB 2194^{T}; Líneas 2-8: cepas representativas de los diferentes serotipos: 11.4 (O1a); 1533 (O1b); RS2 (O2a); 11.29 (O2b); RS6 (O2c); 11.47 (O3); 11.74 (O4); Línea 9: 2576 (cepa chilena BT1/O1a); Línea 10: 8958 (cepa chilena BT1/O1b); Línea 11: 6807 (cepa chilena BT1/O2b); Línea 12: CA2 (cepa portuguesa BT2/O1a); Línea 13: SC0512 (cepa estadounidense BT2/O1b); Línea 14: P20 (cepa finlandesa BT2/O1a); Línea 15: YR9 (cepa inglesa BT2/O1a); Línea 16: 32 (cepa peruana BT2/O1a); Línea 17: A20 (cepa peruana BT1/O1a) Línea 18: PP31 (cepa española BT1/O1a); Línea 19: 217-02 (cepa española BT1/O2b); Línea 20: control negativo.

7. DISCUSIÓN GENERAL

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Desde la descripción de *Yersinia ruckeri* como agente causal de la ERM en la década de los 70, muchos estudios se orientaron a su taxonomía y clasificación intra-específica (Ewing y col., 1978; Stevenson y Airdrie, 1984b; Davies, 1990; Romalde y col., 1993). Sin embargo, desde la primera descripción de cepas no móviles (BT2) en los 80's, pocos estudios se han centrado en su posición taxonómica dentro de la descripción de la especie (Davies y Frerichs, 1989). La re-emergencia de cepas de *Y. ruckeri* inmóviles responsables de los brotes de ERM en peces vacunados a partir de 2001, ha dirigido una atención creciente sobre el BT2, resaltando la importancia de que los estudios incluyan aislados inmóviles para facilitar un mejor diagnóstico y las decisiones de estrategias de control adecuadas.

En este estudio, la caracterización bioquímica realizada incluyó, entre otras, cepas de *Y. ruckeri* pertenecientes al BT2 aisladas de cinco países diferentes (USA, Reino Unido, Finlandia, Portugal y Perú), así como cepas del BT1 aisladas recientemente en Chile de brotes de ERM en salmones vacunados. En general, las características variables entre todas las cepas fueron la movilidad, reacción de Voges-Proskauer (VP), producción de lipasa, fermentación de sorbitol, hidrólisis de gelatina y utilización de citrato. La variabilidad bioquímica de *Y. ruckeri* se ha descrito en muchos estudios (Davies y Frerichs, 1989; Austin y Austin, 2007), alguno de los cuales han resaltado la capacidad de fermentar sorbitol como una característica diferencial para las cepas del serotipo O2 (Davies y Frerichs, 1989). No obstante, esta característica se ha asociado también a cepas de otros serotipos, incluyendo O3 y O4, así como muchos aislados del serotipo O1 (Stevenson y Airdrie 1984b; Romalde y col., 1993; Sousa y col., 2001; Austin y Austin 2007).

A partir de los resultados obtenidos en este estudio, es evidente que además existe heterogeneidad bioquímica entre los aislados inmóviles, lo cual debe ser considerado para evitar diagnósticos erróneos. Las pruebas variables dentro del BT2 fueron específicamente la de reacción de VP y la utilización de citrato. Las contradicciones acerca de las características bioquímicas de aislados inmóviles de *Y. ruckeri* parten de la comparación de aislados procedentes de Inglaterra y de USA (Austin y col., 2003; Arias y col., 2006). Estos dos estudios plantearon que las cepas del Reino Unido tienden a ser VP positivas mientras que los aislados procedentes de USA son VP negativos. Se desconoce si la positividad de VP tiene algún beneficio para los patógenos bacterianos de peces. En el caso de *Aeromonas hydrophila*, se ha observado que las cepas VP positivas producen enterotoxinas citotóxicas diferentes a sus homólogas VP negativos, y que estas citotoxinas están además relacionadas con la aparición de diarrea en humanos (Cumberbatch y col., 1979). Mecanismos similares podrían ocurrir entre los aislados inmóviles VP positivos y negativos de *Y. Ruckeri*. La variación en la prueba VP sugiere que un análisis de los productos finales de fermentación podría ser útil en los estudios taxonómicos y epidemiológicos de *Y. Ruckeri* (Tinsley y col., 2011).

Aunque las reacciones bioquímicas convencionales permiten diferenciar entre cepas de *Y. Ruckeri*, a menudo requieren de mucho tiempo para la preparación de los diversos medios de cultivos y obtención de resultados. Debido a esto, los sistemas miniaturizados como API 20E, API 50CH y API ZYM son cada vez más empleados como primer paso para el diagnóstico de bacterias de peces. En este estudio, todos los perfiles API 20E obtenidos para las diferentes cepas coinciden con los descritos previamente en otros estudios, aun cuando no todos están incluidos en la base de datos del sistema API 20E (Romalde y Toranzo, 1991). Los perfiles obtenidos para la fermentación de carbohidratos

(API 50CH) y actividad enzimática (API ZYM) fueron muy homogéneos entre ambos biotipos, diferenciándose principalmente los perfiles entre los aislados capaces o no de fermentar sorbitol.

Es importante señalar que, aunque los sistemas API 20E y API 50CH resultan útiles y rápidos, deben usarse con precaución en el diagnóstico de infecciones por *Y. Ruckeri*. El uso conjunto de API 20E y API 50CH puede dar una mejor identificación que el empleo del API 20E en solitario. En consecuencia, se deben utilizar ambas pruebas para lograr una identificación más fiable de *Y. Ruckeri*. Por otra parte, se ha recomendado que las pruebas bioquímicas convencionales sean usadas conjuntamente con los sistemas API debido a la discrepancia entre los resultados observados en otros estudios (Romalde y Toranzo, 1991; Topic y col., 2007). En nuestro estudio por ejemplo, se observó que las reacciones de VP en el API 20E a menudo produjeron falsos resultados negativos. La prueba de fermentación del sorbitol debería ser también realizada en paralelo con métodos convencionales cuando se utiliza el sistema API 20E.

Aunque las pruebas bioquímicas permiten identificar los diferentes aislados de *Y. Ruckeri*, las reacciones serológicas han sido claramente más eficaces en identificar positivamente y con rapidez los aislados de los diferentes serotipos de *Y. Ruckeri*. Comúnmente, en las pruebas de aglutinación se utilizan los antígenos O debido a los problemas de reacciones cruzadas que se han observado previamente con antígenos de células enteras (Toranzo y col., 1987). Los resultados descritos por estos autores, junto con los resultados del presente estudio, destacan que los antígenos O son moléculas eficaces para el serotipado de las cepas de *Y. ruckeri*. Todos los aislados analizados se identificaron correctamente mediante las pruebas serológicas empleadas, aunque no se pudiese distinguir entre los biotipos dentro del mismo serotipo. La mayoría de los aislados

inmóviles de origen europeo (Portugal, Reino Unido y Finlandia) incluidos en este estudio pertenecieron al serotipo O1a. Es de resaltar que los aislados del BT2 de origen norteamericano descrito por Arias y col. (2003) correspondieron al serotipo O1b.

El análisis de los patrones de LPS en este estudio permitió evidenciar diferencias entre las cepas según el serotipo. Además, dentro del serotipo O1a, también se observaron diferencias entre el perfil de LPS de las cepas del BT1 y las del BT2. Estos resultados coinciden con los publicados recientemente por Tinsley y col. (2011) quienes, basándose en estas diferencias, proponen la subdivisión del serotipo O1 (según la clasificación de Davies, 1990) en O1a y O1b. Sin embargo, las diferencias en la estructura del antígeno O ya habían sido observadas previamente por Romalde y col. (1993), permitiendo establecer en un nuevo esquema de serotipado, los subgrupos O1a y O1b dentro del serotipo O1a, y los subgrupos O2a, O2b, y O2c dentro del serotipo O2. Aunque estos autores no consideraron el biotipo entre las características fenotípicas de las cepas estudiadas, es de resaltar la necesidad de establecer en adelante, una homogeneidad en la designación de grupos serológicos. Esto evitaría futuras confusiones derivadas de la redundancia de términos dentro de los nuevos esquemas de serotipado que puedan proponerse, con respecto a los anteriores.

Las diferencias en la estructura antigénica o inmunogénica de los LPS se deben a la variabilidad en las cadenas de polisacáridos. Zamze y Morton (1987) demostraron que los diferentes serotipos de *Haemophilus influenzae* presentan diferencias cuantitativas en la composición química de sus LPS, que se correlacionan con su especificidad antigénica y su movilidad en geles de poliacrilamida. Esto sugiere que para subdividir con mayor precisión el serotipo O1a, podría considerarse el estudio detallado de la estructura química de sus LPS

además de sus características antigénicas. Sobre esta base, se podría suponer que las diferencias en la composición química y del perfil de LPS pueden relacionarse con la virulencia del patógeno o la respuesta inmune del huésped hacia esta molécula.

Los resultados obtenidos en esta memoria evidenciaron que el BT2 de *Y. ruckeri* puede también estar asociado con el grupo serológico O1b, destacando la existencia de nuevos grupos clonales y la necesidad de revisión del sistema clonal de Davies (1991a). Este autor sugirió que, los brotes de la ERM se asociaban sólo con los grupos clonales 2 (incluye cepas del BT2) y 5 (incluye cepas del BT1) pertenecientes exclusivamente al serotipo O1a. Así, las cepas del BT2 aisladas en Reino Unido y resto de Europa estaban clasificadas con el OMP tipo 2 y el serotipo O1a es decir, dentro del grupo clonal 2. Otros autores también han señalado esta necesidad en base a la existencia de cepas inmóviles dentro del serotipo O4 y en aislados no serotipables (Tinsley y col., 2011). Por otra parte, debido a que la obtención de los perfiles de OMP requiere mucho tiempo y a que la expresión de estas proteínas está sujeta a cambios en las condiciones de cultivo, estos últimos autores también sugirieron excluir el tipado de OMPs de los procedimientos de diagnóstico. Aunque la técnica es fácilmente replicable en los laboratorios, los estudios genéticos han proporcionado mejores evidencias sobre la relación entre los aislados *Y. ruckeri* (Wheeler y col., 2009).

El genotipado por ERIC-PCR permitió diferenciar en este estudio, 7 patrones entre todos los aislados de *Y. ruckeri*, consistentes en 8-10 bandas entre 250 y 1700 bp. Por otra parte, los 4 perfiles obtenidos por REP-PCR fueron más homogéneos, consistiendo entre 9 a 11 bandas amplificadas que van desde 150 hasta 2600 bp. El empleo de otras técnicas moleculares, como los estudios de perfiles de plásmidos, PFGE y MLEE, ya había permitido estudiar la variabilidad

genética de cepas de *Y. ruckeri,* obteniendo importantes relaciones entre cepas similares responsables de brotes de ERM en los cultivos de salmónidos (De Grandis y Stevenson, 1982; Schill y col., 1984; García y col., 1998; Lucangeli y col., 2000; Wheeler y col., 2009). Aunque otros autores han informado de la variabilidad genética entre las cepas de *Y. ruckeri* del BT1 y el BT2 utilizando PFGE (Wheeler y col., 2009; Ström-Bestor y col., 2010), la variabilidad genética observada entre las cepas de *Y. ruckeri* evaluadas en este estudio se relacionó principalmente con el serotipo.

En nuestro estudio se ha encontrado una alta diferenciación a nivel intraespecífico entre las cepas de *Y. ruckeri,* aisladas recientemente de brotes de ERM en diferentes zonas geográficas, utilizando la combinación de diferentes técnicas de tipado. Dentro de las cepas del serotipo O1a esta variabilidad permitió observar diferentes grupos entre los aislados del BT1 y BT2. También se observaron ambos biotipos entre los aislados del serotipo O1b. Estos resultados evidencian una alta discriminación entre aislados cuando se analizan conjuntamente los resultados del API 20E, los perfiles LPS y de OMP, destacando éstos como métodos de tipado valiosos, especialmente en combinación con cualquier de las otras técnicas de genotipado aplicadas en este estudio (ERIC-PCR y REP-PCR). Además, se observó una relación entre grupos con características similares y algunas especies de peces y/o predominio en algunas áreas geográficas, lo que concuerda con resultados similares obtenidos en otros estudios (Wheeler y col., 2009; Welch y col., 2011). Nuestros resultados destacan además la utilidad de este enfoque polifásico en los estudios de diversidad bacteriana con fines epidemiológicos.

En este estudio hemos desarrollado un protocolo de qPCR basado en el gen *rec*A, que resultó ser eficaz en la detección y medición cuantitativa de *Y. ruckeri*,

no sólo a partir de cultivos puros del patógeno, sino también a partir de homogenizados de tejidos de peces, incluyendo la sangre. La presencia de la bacteria no siempre se pudo relacionar con mortalidad en peces inoculados ya que, las mortalidades no ocurrieron hasta que las cargas bacterianas en los tejidos superaban 10^6 ufc/g^{-1}. Estos resultados demuestran la utilidad de éste ensayo de qPCR para detectar las variaciones de los niveles de *Y. ruckeri* en los tejidos del pez, y sugieren que este nivel de carga bacteriana puede ser un umbral crítico entre un estado epizoótico o portador.

Estudios recientes se han centrado en el desarrollo de nuevas técnicas eficaces en la detección y diferenciación de fenotipos emergentes y de los diferentes serotipos de *Y. ruckeri.* Welch y col. (2011) se basaron en técnicas de PCR para desarrollar diferentes ensayos para la detección de los cuatro alelos mutantes identificados como causantes de la pérdida de motilidad en las cepas del BT2 de *Y. ruckeri* circulantes en Europa y los Estados Unidos. Ostrowski (2012) diseñó un protocolo de PCR capaz de la discriminación rápida y reproducible del serotipo O1 de *Y. ruckeri* utilizando los genes *wzx* y *wzy* implicados en la síntesis de antígeno O. Estos avances han permitido la detección rápida y precisa de *Y. ruckeri* en muestras de peces, aunque por otro lado, carecen de la capacidad de detectar y cuantificar la carga bacteriana en muestras no letales.

Por lo tanto, el protocolo de qPCR desarrollado en el presente estudio ofrece una alternativa útil desde un enfoque microbiológico, para el diagnóstico rápido, específico, y no letal de *Y. ruckeri* en los peces infectados, así como para la detección potencial de poblaciones asintomáticas o portadoras dentro de los cultivos. Este ensayo, aunque no de manera discriminante, también resultó útil para la detección de todos los biotipos y serotipos de *Y. ruckeri* incluidos en este estudio, lo que demuestra que este protocolo de qPCR puede ser potencialmente

adecuado para su uso en aquellas áreas donde el BT2 y otros serotipos están siendo descritos como un problema creciente.

Las técnicas de caracterización fenotípica y serológica resultan útiles en el diagnóstico de patógenos bacterianos, sin embargo limitan la comprensión de la genética de poblaciones en bacterias. En nuestro estudio, se utilizó la técnica de MLST para analizar la población de *Y. ruckeri,* logrando obtener nueva información sobre su estructura genética, así como sobre los mecanismos asociados a la aparición de los diferentes linajes que se han extendido con éxito a nivel mundial. En primer lugar, se evidenció que la recombinación contribuye 7,4 veces más que las mutaciones puntuales a la variación genética entre las secuencias, desempeñando un papel más importante en la generación y mantenimiento de la diversidad dentro de *Y. ruckeri*. Además, se identificaron 30 tipos de secuencias (STs), que se agruparon en dos complejos clonales (CC) separados (cada uno con su respectivo ancestro común) y tres STs únicos. Nuestros resultados constituyen así la primera demostración de que *Y. ruckeri* no presenta una estructura poblacional clonal si no que se adecúa a un modelo epidémico, en el que clones emergentes bien adaptados se mantienen y además se distribuyen ampliamente.

Este modelo de estructura poblacional también es consistente con la epidemiología de la enfermedad, que sugiere que la ERM comenzó como una enfermedad aislada geográficamente llegando a ser ampliamente difundida con relativa rapidez. El CC1 se vinculó casi exclusivamente a los brotes asociados con el cultivo de los salmónidos en el mundo desde 1970, mientras que el CC2 estuvo restringido geográficamente a USA, Dinamarca y Canadá (desde 1980 a 1987), siendo en su mayoría aislados de peces no salmónidos o de fuentes ambientales. Estos resultados podrían reflejar una adaptación por especialización de nicho,

apoyando la hipótesis de que la difusión de *Y. ruckeri* se produjo cuando este patógeno encontró un nicho alternativo (cultivo intensivo de la trucha arcoíris) más que por rutas preferenciales de transmisión (Welch y col., 2011).

A pesar de las limitaciones del MLST para determinar relaciones genéticas exactas entre los diferentes aislados, estos datos representaron un valioso recurso para obtener información a nivel poblacional. Desde este enfoque, los resultados de nuestro estudio permitieron confirmar que la aparición y dispersión de la ERM en Europa y América del Sur durante las últimas décadas ha sido el resultado de dos eventos independientes y paralelos. En primer lugar, que los factores intrínsecos tales como la diferenciación genética de la población, la especialización de nicho y/o el aislamiento por distancia, han jugado un papel importante en la diversificación y persistencia del patógeno; y en segundo lugar, que la distribución de la ERM a nivel global ha sido facilitada por la acción humana, a través de la transferencia de peces portadores de *Y. ruckeri*.

Una de las principales ventajas de los enfoques basados en la secuenciación es que los datos pueden ser alojados en Internet (http://pubmlst.net; www.mlst.net) y mantenerse constantemente actualizados (Maiden y col., 1998). El esquema de MLST que hemos establecido para *Y. ruckeri* en este estudio es de libre acceso y se encuentra actualmente alojado en una de las base de datos públicas (http://pubmlst.org/yruckeri/). A medida que se incluyan nuevas secuencias, podrían establecerse nuevos STs, y también es probable que las conclusiones relativas a los ancestros comunes y complejos clonales tengan que ser modificados. Partiendo de la existencia de esta base de datos, las futuras investigaciones basadas en el genotipado de *Y. ruckeri* podrían además orientarse al uso de genes que están bajo una mayor presión selectiva, tales como genes de virulencia. El desarrollo de esquemas de MLST en combinación con genes

virulencia de la flagelina (MLST-V) ha permitido identificar diferencias sero-específicas en aislados de *Salmonella* (Tankouo-Sandjong y col., 2007). Estos autores observaron que la transferencia lateral del gen *fli*C transformaban una serovariedad en otra nueva, y que utilizando técnicas de serotipado no se reconocían estos cambios en el serotipo. Futuros estudios sobre las características antigénicas de *Y. ruckeri* podrían ayudar a identificar antígenos claves que pueden ser adecuados para su incorporación en un estudio MLST-V.

Los análisis filogeográficos realizados en este estudio evidenciaron tres poblaciones genéticas ancestrales de *Y. ruckeri* que pudieron haber surgido, coexistido y extendido separadamente. El grupo ancestral más importante incluyó cepas aisladas principalmente de *O. mykiss* en USA, Perú, Reino Unido, Finlandia y España. El segundo grupo ancestral se definió de la mayoría de las cepas del serotipo O1b aisladas de *S. salar* en Chile, relacionadas también con otras cepas del serotipo O1b aisladas de *O. mykiss* en el Reino Unido, y en menor proporción con aislados de Portugal. El tercer grupo ancestral compiló cepas de *Y. ruckeri* aisladas en Canadá, Portugal y Dinamarca no pertenecientes al serotipo O1a. La cría de truchas realmente se remonta a hace más de 400 años en Europa, alrededor de hace 150 años en los USA, y cerca de 100 años en el Suramérica (Hinshaw, 1990), lo cual apoyaría la existencia de estos grupos genéticos ancestrales asociados a esta actividad. Las señales de desequilibrio genético observadas en la población indican que ha habido además varios cambios en la población asociados a la expansión demográfica y espacial a través del tiempo. Hemos estimado que estos cambios pudieron haber ocurrido como mínimo hace 800 años variando entre las diferentes áreas, y que en algunas regiones como Chile, Canadá y Portugal, la diversidad genética mostró indicios de aislamiento por distancia con respecto al resto de los países involucrados en este estudio.

Por otra parte, hemos determinado que la tasa evolutiva promedio de *Y. ruckeri* es de 2.5 x 10^{-5} sustituciones por nucleótido/sitio/año, apuntando a que este patógeno puede evolucionar más rápido de lo comúnmente observado en otras bacterias. Se ha descrito que *Escherichia coli* tiene una tasa evolutiva de 3 x 10^{-8} sustituciones por nucleótido/sitio/año (Guttman y Dykhuizen, 1994). Pocas bacterias, incluyendo otra especie del género *Yersinia*, *Y. pestis*, han mostrado tasas evolutivas más altas que la de *E. coli* (Morelli y col., 2010).

La fuerte estructuración de los linajes *Y. ruckeri* entre los diferentes países observada en nuestro estudio, sugiere que, la evolución *in situ* también ha desempeñado un papel importante en el mantenimiento de la ERM en el mundo. Desde este punto de vista, el mantenimiento enzoótico de *Y. ruckeri* podría ser, después de la migración extensiva, otra vía alternativa, propiciada también por la acuicultura, que explicaría la especificidad de cepas observada en países como Chile, Canadá y Portugal.

Los análisis de la dinámica poblacional de *Y. ruckeri* revelaron en este estudio una sincronía con la historia de la ERM a nivel global. La observación de un rápido aumento en la diversidad genética entre 1950 y 1975, correspondió con el período de emergencia de los principales linajes que se fueron generalizando probablemente a través del cultivo de salmónidos en toda América del Norte y Europa (Austin y Austin, 2007). Seguidamente, este crecimiento experimentó un fuerte declive entre 1980 y 2000, causado probablemente, por la implementación de la vacunación como estrategia de control contra *Y. ruckeri*. Coincidiendo con la reciente emergencia del BT2 y otras variantes serológicas, causantes de los nuevos brotes de ERM, a partir de 2001 (Austin y col., 2003; Fouz y col., 2006; Arias y col., 2007), se produce nuevamente un aumento de diversidad genética, esta vez de manera abrupta. Estos hallazgos confirman que, la combinación de

factores ecológicos y evolutivos han propiciado el rápido aumento en la diversidad, es decir, la propagación de la enfermedad a través de grandes poblaciones de salmónidos inmunológicamente desprotegidos, junto con las tasas relativamente altas de sustitución de nucleótidos, así como la presión de la selección sobre la población de *Y. ruckeri.*

La reconstrución del proceso de difusión de *Y. ruckeri* entre diferentes países a partir de la información temporal y espacial de cada una de las secuencias estudiadas permitió determinar a USA como la fuente de origen del proceso de dispersión de la ERM. La mayoría de las vías de transmisión inferidas entre USA y los países europeos se apoyaron estadísticamente, lo que no ocurrió para la transmisión hacia América del Sur. Por el contrario, se establecieron varios vínculos significativos entre países europeos como Reino Unido, Dinamarca y Noruega con Chile. La mayoría de las relaciones epidemiológicos establecidas representan enlaces entre distintos continentes, lo que nuevamente, apoya la propagación a larga distancia de *Y. ruckeri* a través de movimiento peces durante la expansión de la salmonicultura.

El hecho de que los ancestros comunes más recientes para el BT2 de *Y. ruckeri* en el Reino Unido y USA existieran separadamente en cada uno de estos países, confirma la hipótesis de la emergencia independiente de este fenotipo en Norte América y Europa (Wheeler y col., 2009; Welch y col., 2011). Por otro lado, la falta de una vinculación epidemiológica significativa entre USA con Portugal y Perú, así como de Finlandia con cualquier otra área, apoya también la aparición independiente de diferentes linajes de BT2 de *Y. ruckeri* en los diferentes continentes.

Este estudio proporciona evidencia de que hay una tendencia creciente al aumento del BT2 y en la virulencia dentro del serotipo O1b de *Y. ruckeri* (como

en USA, Reino Unido y en Chile), que podría seguir causando problemas en el futuro. Hemos calculado un tiempo de coalescencia para la población actual de *Y. ruckeri* de hace aproximadamente 600 años. Cabe destacar que desde entonces, ésta especie se ha diferenciado en siete grupos antigénicamente distintos (Romalde y col., 1993). Es posible que la alta tasa de cambio evolutivo determinada en *Y. ruckeri*, pueda en parte explicar el alto nivel de diversidad antigénica alcanzado por este patógeno y, a su vez, la resistencia desarrollada en menos de treinta años a la vacuna comercial contra la ERM. De ésta manera, podemos plantear la hipótesis de que la emergencia y aumento de los nuevos casos de la ERM en todo el mundo puede ser una consecuencia natural de la acuicultura intensiva, debida a la sustitución de cepas mediada por acción de la vacunación.

La acuicultura intensiva puede ser una razón de la evolución de la virulencia de patógenos. Pulkkinen y col. (2010) plantearon la hipótesis de que la mayor incidencia de *Flavobacterium columnare* era debida a un cambio evolutivo en la virulencia causada por la acuicultura intensiva en Finlandia. Este cambio también podría ser resultado de cambios en el ecosistema causados por la acuicultura intensiva o posiblemente una combinación de los dos factores. Estos autores también sugirieron que la presencia en un área de varias poblaciones de bacterias genéticamente distintas puede favorecer la virulencia si las cepas virulentas tienen una ventaja competitiva. Bachrach y col. (2001) identificaron un nuevo serogrupo en *Streptococcus iniae* a partir de brotes de la enfermedad, causadas por este patógeno, en peces previamente vacunados. Los nuevos aislados, a diferencia de los aislados anteriores, eran ADH (arginina dihidrolasa) negativos. Eyngor y col. (2004) describieron procesos similares en *Lactococcus garvieae*, planteándose la hipótesis de que la presión selectiva inducida por la vacunación específica contra

L. garvieae, causaba la variación serológica de la bacteria a través de cambios en la composición capsular.

Según la teoría del reemplazo de cepas mediado por la vacunación (Martcheva y col., 2008), a medida que la prevalencia de cepas del BT1 disminuya, la prevalencia de BT2 aumentaría, posiblemente debido a que el BT2 no tiene que competir con BT1 ya que la vacuna monovalente mantiene su incidencia a niveles bajos. De esta manera, el tratamiento contra la enfermedad puede seleccionar de manera positiva las formas más virulentas, dependiendo de la periodicidad del tratamiento en relación con la transmisión de bacterias. Los experimentos de supervivencia de *Y. ruckeri* en el agua y el sedimento han demostrado que el patógeno es capaz de sobrevivir durante más de cuatro meses en el ambiente (Romalde y col., 1994). El estudio también demostró que la bacteria posee la capacidad de permanecer en estado durmiente ("starvation") cuando se encuentra en condiciones adversas (disminuyendo su tamaño y actividad hasta que las condiciones desfavorables desaparecen), manteniendo intacta su virulencia. Podría ser posible que con el tiempo, el efecto de la selección, de la acuicultura y la vacunación, haya causado que las cepas virulentas de *Y. ruckeri* llegaran a ser más saprofitas. Nuevamente, la investigación de los compuestos excretados por aislados de *Y. ruckeri* de diferentes serotipos y biotipos serían útil para determinar si estos nuevos biotipos están produciendo sustancias diferentes que pueden darles una ventaja selectiva sobre las cepas móviles.

8. CONCLUSIONES

A partir de los resultados obtenidos en cada uno de los capítulos de esta memoria podemos deducir las siguientes conclusiones:

1. La caracterización fenotípica, serológica y genética de las cepas de *Yersinia ruckeri* demuestra que:

a) Las diferentes cepas de *Y. ruckeri* aisladas recientemente de brotes de ERM en peces vacunados, presentan una gran variabilidad a nivel de sus características bioquímicas, serológicas y genéticas.
b) Se han desrito por primera vez cepas de *Y. ruckeri* del biotipo 2 aislados de brotes de la ERM en Portugal y Suramérica.
c) Los aislados pertenecientes al serotipo O1b, tanto móviles como inmóviles, representan una variante emergente para la aparición de brotes de yersiniosis en peces vacunados.
d) El análisis de las pruebas API 20E, los perfiles LPS y de OMP en combinación con técnicas de genotipado como ERIC-PCR o REP-PCR, resultan altamente discriminantes en el estudio de la diversidad de *Y. ruckeri* con fines epidemiológicos.

2. Los estudios de estructura poblacional, historia demográfica y filogeografía de *Yersinia ruckeri* permiten concluir que:

a) Los genes *dna*J, *gln*A, *gyr*B, Y-HSP60, *rec*A and *thr*A son adecuados para el estudio de la filogénia y la estructura de la población de *Y. ruckeri.*
b) La estructura de la población de *Y. ruckeri* está compuesta por dos complejos clonales y tres tipos de secuencias únicas, y sigue un

modelo de expansión epidémico.

c) La población de *Y. ruckeri* ha experimentado cambios en la población (demográficos y espaciales) tanto antiguos (que pudieron ocurrir como mínimo hace 800 años), como recientes (desde hace 50 años) inducidos posiblemente por procesos biológicos en el pasado y, más recientemente, por procesos de adaptación forzada por el auge de la acuicultura.

3. Los análisis de la divergencia y la reconstrucción de la transmisión histórica y espacial de *Y. ruckeri* permitieron inferir que:

a) *Y. ruckeri* presenta una alta tasa evolutiva con un promedio de 2.5 x 10^{-5} sustituciones por nucleótido/sitio/año para los genes *dna*J, *gln*A, *gyr*B, Y-HSP60, *rec*A and *thr*A.

b) La especie *Y. ruckeri* se compone de múltiples linajes que han surgido de forma independiente, a partir de ancestros diferentes, a lo largo del tiempo.

c) USA fue la fuente de origen del proceso de dispersión de la ERM y que la propagación a larga distancia de *Y. ruckeri* se realizó a través del movimiento de peces durante la expansión de la salmonicultura.

4. El protocolo de qPCR en tiempo real desarrollado en este trabajo posee una gran especificidad y alta sensibilidad para la detección y quantificación de *Y. ruckeri en* tejidos de la trucha arcoíris, siendo de gran utilidad como herramienta no letal, para el diagnóstico de la ERM, tanto en casos clínicos como de estados portadores.

9. REFERENCIAS BIBLIOGRÁFICAS

9. Referencias Bibliográficas

Abbass, A., Aharifuzzaman, A. A. and Austin, B. (2010) Cellular components of probiotics control *Yersinia ruckeri* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis. 33: 31-37.

Adams, A., Thompson, K. D., Morris D., Farias, C., Chen, S. C. (1995) Development and use of monoclonal antibody probes for immunohistochemistry, ELISA and IFAT to detect bacterial and parasitic fish pathogens. Fish Shellfish Immunol. 5: 537-547.

Aleksic, S., Steigerwalt, A. G., Bockemuhl, J., Huntley-Carter, G. P. and Brenner, D. J. (1987) *Yersinia rodhei* sp. nov. isolated from human and dog feces and surface water. Int. J. Syst. Bacteriol. 37: 327-332.

Altinok, I. (2004) The infectious route of *Yersinia ruckeri* is affected by salinity. Bull. Eur. Ass. Fish Pathol. 24: 253-259.

Altinok, I., Capkin, E. and Kayis, S. (2008) Development of multiplex PCR assay for simultaneous detection of five bacterial fish pathogens. Vet. Microbiol. 131: 332-338.

Altinok, I., Grizzle, J. M. and Liu, Z. (2001) Detection of *Yersinia ruckeri* in rainbow trout blood by use of the polymerase chain reaction. Dis. Aquat. Org. 44: 29-34.

Amend, D. F., Johnson, K. A., Croy, T. R. and McCarthy, D. H. (1983) Some factors affecting the potency of *Yersinia ruckeri* bacterins. J. Fish Dis. 6: 337-344.

Ammendola, S., Pasquali, P., Pistoia, C., Petrucci, P., Petrarca, P., Rotilio, G. and Battistoni, A. (2007) High-affinity $Zn2^{+}$ uptake system ZnuABC is required for bacterial zinc homeostasis in intracellular environments and contributes to the virulence of *Salmonella enterica*. Infect. Immun. 75:

5867-5876.

An, Y. H. F. and Friedman, R. J. (2000) Handbook of bacterial adhesion: principles, methods, and applications. Eds. Humana Press. Torowa, NJ, USA.

Argenton, F., De Mas, S., Malocco, C., Dalla Valle, L. and Giorgetti, G. (1996). Use of random DNA amplification to generate specific molecular probes for hybridization tests and PCR-based diagnosis of *Yersinia ruckeri*. Dis. Aquat. Org. 24: 121-127.

Arias, C. R., Olivares-Fuster, O., Hayden, K., Shoemaker, C. A., Grizzle, J. M. and Klesius, P. H. (2007) First report of *Yersinia ruckeri* biotype 2 in the USA. J. Aquat. Anim. Health 19: 35-40.

Austin, B. and Austin, D. A. (2007) Bacterial Fish Pathogens: Disease in Farmed and Wild Fish (4th ed.), Springer Praxis, Chichester.

Austin, D. A., Robertson, P. A. W. and Austin, B. (2003) Recovery of a new biogroup of *Yersinia ruckeri* from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum). Syst. Appl. Microbiol. 26: 127-131.

Avci, H. and Birincioglu, S. S. (2005) Pathological findings in rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) experimentally infected with *Yersinia ruckeri*. Turk. J. *Vet.* Anim. Sci. 29: 1321-1328.

Avise, J. (2000) Phylogeography: the history and formation of species. Cambridge, Massachussets: Harvard University Press. pp 4-37.

Baba, T., Inamura, J. and Izawa, K. (1988) Immune protection in carp, *Ciprinus carpio* L., after immunization with Aeromonas hydrophila through lipopolysaccharide. J. Fish Dis. 11: 237-244.

Bachrach, G., Zlotkin, A., Hurvitz, A., Evans, D. L. and Eldar, A. (2001) Recovery of *Streptococcus iniae* from diseased fish previously

vaccinated with a *Streptococcus* vaccine. Appl. Environ. Microbiol. 67: 3756-3758.

Bangert, R. L., Ward, A. C. S., Stauber, E. H., Cho, B. R. and Widders, P. R. (1988) A survey of the aerobic bacteria in the feces of captive raptors. Avian Dis. 32: 52-62.

Beheregaray, L. and Caccone, A. (2007) Cryptic Biodiversity in a Changing World. J. Biol. 6: 1-5.

Bercovier, H., Steigerwalt, A. G., Guiyoule, A., Huntley-Carter, G. and Brenner D. J. (1984) *Yersinia aldovae* (formerly *Yersinia enterolitica* like Group X2 a new species of *Enterobacteriaceae* isolated from aquatic ecosystems. Int. J. Syst. Bacteriol. 34: 166-172.

Bercovier, H., Ursing J., Brenner, D.J., Steigerwalt, A. G., Fanning, G. R., Carter, G. P. and Mollaret, H. H. (1981) In: Validation of the publication of new names and new combinations previously effectively published outside the IJSB. List Nº 6. Int. J. Syst. Bacteriol. 31: 215-218.

Bos, M. P., Robert, V. and Tommassen, J. (2007) Biogenesis of the Gram-negative bacterial outer membrane. Annu. Rev. Microbiol. 61: 191-214.

Bottone, E., Bercovier, H. and Mollaret, H. H. (2005) Genus XLI. Yersinia *Van loghem* 944, 15^{AL}. In*:* Brenner, D. J., Krieg, N. R. and Staley, J. T. (eds.) Bergey's manual of systematic bacteriology. Vol. 2, Part B. Springer Inc., New York. p. 838-848.

Boyd, E. F., Nelson, K., Wang, F. S., Whittam, T. S. and Selander, R. K. (1994) Molecular genetic basis of allelic polymorphism in malate dehydrogenase (*mdh*) in natural populations of *Escherichia coli* and *Salmonella enterica*. Proc. Nat. Acad. Sci. USA. 91: 1280-1284.

Bravo, S. (1993) Diseases Reported in Pen Reared Salmonids from Chile. FHS/AFS Newsletter. 21: 3.

Bravo, S. and Kojagura, V. (2004) First isolation of *Yersinia ruckeri* from rainbow trout (*Oncorhynchus mykiss*) in Peru. Bull. Eur. Ass. Fish Pathol. 24: 104-108.

Brenner, D. J., Bercovier, H., Ursing, J., Alonso, J. M., Steigerwalt, A.G., Fanning, G. R., Carter, G. P. and Mollaret, H. H. (1980) *Yersinia intermedia*: a new species of *Enterobacteriaceae* composed of rhamnose-positive, melibiose-positive, raffinose-positive strains (formerly called *Yersinia enterocolitica* or *Yersinia enterocolitica*-like). Curr. Microbiol. 4: 207-212.

Bruno, D. W. and Munro, A. L. S. (1989) Immunity in Atlantic salmon, *Salmo salar* L., fry following vaccination against *Yersinia ruckeri*, and the influence of body weight and infectious pancreatic necrosis virus (IPNV) on the detection of carriers. Aquaculture. 81: 205-211.

Bullock, G. L. and Cipriano, R. C. (1990) Enteric Redmouth Disease of Salmonids. US Fish & Wildlife Publications. 131: 1-4.

Bullock, G. L., Maestrone, G., Starliper, C. and Schill, B. (1983) Potentiated sulphonamide therapy of enteric redmouth disease. Can. J. Fish. Aquat. Sci. 40: 101-102.

Bullock, G., Stuckkey, H. and Shotts, E. (1978) Enteric redmouth bacterium: comparison of isolates from different geographic areas. J. Fish Dis. 1: 351-356.

Busch, R. A. (1973) The serological surveillance of salmonid populations for presumptive evidence of specific disease association. Ph.D. Thesis, University of Idaho, Moscow.

Busch, R. A. (1978) Enteric redmouth disease (Hagerman strain). Mar. Fish. Rev. 40: 42-51.

Busch, R. A. (1982) Enteric redmouth disease (*Yersinia ruckeri*). In*:* Anderson, D. P., Dorson, M., Dubourget, P. (eds.) Antigens of fish pathogens. Development and production of vaccines and serodiagnostics. Lyon: Symposium International de Taloires.

Busch, R. A. and Ling, A. J. (1975) Establishment of an asymptomatic carrier state infection of enteric redmouth disease in rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can. 32: 2429-2432.

Campos, N. R. (1995) El lipopolisacarido bacteriano: una potente endotoxina con múltiples actividades biológicas. Rev. Cost. Cienc. Méd. 16: 71-84.

Canhos, V. P., Souza, S., Giovanni, R. and Canhos, D. A. L. (2004) Global biodiversity informatics: setting the scene for a "new world" of ecological modeling. Biodiv. Inform. 1: 1-13.

Capkin, E. and Altinok, I. (2009) Effects of dietary probiotic supplementations on prevention/treatment of yersiniosis disease. J. Appl. Microbiol. 106: 1147-1153.

Chen, Q., Yan, Q., Wang, K., Zhuang, Z. and Wang, X. (2008) Portal of entry for pathogenic *Vibrio alginolyticus* into large yellow croaker *Pseudosciaena crocea*, and characteristics of bacterial adhesion to mucus. Dis. Aquat. Org. 80: 181-188.

Cipriano, R. and Ruppenthal, T. (1987) Immunization of salmonids against *Yersinia ruckeri*: Significance of humoral immunity and cross protection between serotypes. J. Wildlife Dis. 23: 545-550.

Coquet, L., Cosette, P., Quillet, L., Petit, F., Junter, G.-A. and Jouenne, T. (2002) Occurrence and phenotypic characterization of *Yersinia ruckeri* strains with

biofilm-forming capacity in a rainbow trout farm. Appl. Environ. Microbiol. 68: 470-475.

Cornelis, G. R. (1998) The *Yersinia* deadly kiss. J. Bacteriol. 180: 5495-5504.

Costa, A. A., Leef, M. J., Bridle, A. R. , Carson, J. and Nowak, B. F. (2011) Effect of vaccination against yersiniosis on the relative percent survival, bactericidal and lysozyme response of Atlantic salmon, *Salmo salar*. Aquaculture. 315: 201-206.

Crane, J. K., Naeher, T. M., Shulgina, I., Zhu, C. and Boedeker, E. C. (2007) Effect of zinc in Enteropathogenic *Escherichia coli* infection. Infect. Immun. 75: 5974-5984.

Cumberbatch, N., Gurwith, M. J., Langston, C., Sack, R. B. and Brunton, J. L. (1979) Cytotoxic enterotoxin produced by *Aeromonas hydrophila*: relationship of toxigenic isolates to diarrheal disease. Infect. Immun. 23: 829-837.

Dahiya, I. and Stevenson, R. M. W. (2010) The UvrY response regulator of the BarA-UvrY two-component system contributes to *Yersinia ruckeri* infection of rainbow trout (*Oncorhynchus mykiss*). Arch. Microbiol. 192: 541-547.

Dalsgaard, I. J., From, J. and Horlyck, V. (1984) First observations of *Y. ruckeri* in Denmark. Bull. Eur. Ass. Fish Pathol. 4: 10.

Daly, J. G., Lindvik, B. and Stevenson, R. M. W. (1986) Serological heterogeneity of recent isolates of *Yersinia ruckeri* from Ontario and British Columbia. Dis. Aquat. Org. 1: 151-152.

Davies, R. L. (1990) O-serotyping of *Yersinia ruckeri* with special emphasis on European isolates. Vet. Microbiol. 22: 299-307.

Davies, R. L. (1991a) Clonal analysis of *Yersinia ruckeri* based on biotypes, serotypes and outer membrane protein types. J. Fish Dis. 14: 221-228.

Davies, R. L. (1991b). *Yersinia ruckeri* produces four iron-regulated outer membrane proteins but does not produce detectable siderophores. J. Fish Dis. 14: 563-570.

Davies, R. L. and Frerichs, G. N. (1989) Morphological and biochemical differences among isolates of *Yersinia ruckeri* obtained from wide geographical areas. J. Fish Dis. 12: 357-365.

Davis, B. (1990) Bacterial Architecture. In: Davis, B., Dulbecco, R., Eisen H. and Ginsberg, H. (eds.). Microbiology, 4th Edition. J. B. Lippincott Co. Philadelphia, pp. 21- 50.

Dear, G. (1988) *Yersinia ruckeri* isolatedf rom Atlantic salmon in Scotland. Bull. Eur. Ass. Fish Pathol. 8: 18-20.

De Grandis, S. A., Krell, P. J., Flett, D. E. and Stevenson, R. M. W. (1988) Deoxyribonucleic-acid relatedness of serovars of *Yersinia ruckeri*, the enteric redmouth bacterium. Int. J. Syst. Bacteriol. 38: 49-55.

De Grandis, S. A. and Stevenson, R. M. W. (1982) Variations in plasmid profiles and growth-characteristics of *Yersinia ruckeri* strains. FEMS Microbiol. Lett. 15: 199-202.

De Grandis, S. A. and Stevenson, R. M. W. (1985) Antimicrobial susceptibility patterns and R plasmid-mediated resistance of the fish pathogen *Yersinia ruckeri*. Antimicrob. Agents Chemother. 27: 938-942.

De La Cruz, J. A., Rodriguez, A., Tejedor, C., de Lucas, E. and Orozeo, L. R. (1986) Isolation and identification of *Yersinia ruckeri*, causal agent of the

enteric redmouth disease (ERM) for the first time in Spain. Bull. Eur. Ass. Fish Pathol. 6: 43-44.

Dulin, M. P., Huddleston, T. Larson, R. E. and Klontz, G. W. (1976) Enteric redmouth disease. Univ. Idaho, Moscow, For. Wildl. Range Exp. Stn., Contrib. No. 16. p.15.

Effendi, I. and Austin, B. (1995) Uptake of *Aeromonas salmonicida* by Atlantic salmon (*Salmo salar*). Bull. Eur. Ass. Fish Pathol. 15: 115-118.

Evans, T., Ind, A., Komitopoulou, E. and Salmond, G. (2010) Phage selected lipopolysaccharide mutants of *Pectobacterium atrosepticum* exhibit different impacts on virulence. J. Appl. Microbiol. 109: 505-514.

Evenhuis, J., LaPatra, S., Verner-Jeffreys, D., Dalsgaard, I. and Welch, T. (2009) Identication of flagellar motility genes in *Yersinia ruckeri* by transposon mutagenesis. Appl. Envirom. Microbiol. **7**5: 6630-6633.

Ewing, W., Ross, A., Brenner, D. and Fanning, G. (1978) *Yersinia ruckeri* sp. nov., the redmouth (RM) bacterium. Int. J. Syst. Evol. Microbiol. 28: 37-44.

Eyngor, M., Zlotkin, A., Ghittino, C., Prearo, M., Douet, D.-G., Chilmonczyk, S. and Eldar, A. (2004) Clonality and diversity of the fish pathogen *Lactococcus garvieae* in Mediterranean countries. Appl. Environ. Microbiol. 70: 5132-5137.

Fallman, M. and Gustavsson, A. (2005) Cellular mechanisms of bacterial internalization counteracted by *Yersinia*. Int. Rev. Cytol. 246: 135-188.

FAO (2006) Topics and Issues Fact Sheet. State of World Fisheries: Systems, Food and Agriculture Organization of the United Nations, Rome.

FAO (2007) Fisheries Statistics: Nations, Food and Agriculture Organization of the United Nations, Rome.

Faraldo-Gómez, J. D. and Sansom, M. S. P. (2003) Acquisition of siderophores in Gram-negative bacteria. Nat. Rev. Mol. Cell. Biol. 4: 105-116.

Farmer, J. J. III, Davies, B. R., Hikman-Brenner, F. W., McWhoter, A., Huntley-Carter, G. P., Asbury, M. A., Riddle, M. and Wathen-Grady, A. (1985) Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. J. Clin. Microbiol. 21: 46-76.

Feil, E., Li, B. C., Aanensen, D. M., Hanage, W. P. and Spratt, B. G. (2004) eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J. Bacteriol. 186: 1518-1530.

Feng, L., Reeves, P. R., Lan, R. Ren, Y., Gao, C., Zhou Z., Ren, Y., Cheng, J., Qian W., Li, D. and Wang, L. (2008) A recalibrated molecular clock and independent origins for the cholera pandemic clones. PLoS ONE 3: e4053.

Fernández, L., López, J. R., Secades, P., Menéndez, A., Márquez, I. and Guijarro, J. A. (2003) *In vitro* and *in vivo* studies of the *yrp1* protease from *Yersinia ruckeri* and its role in protective immunity against enteric red mouth disease of salmonids. Appl. Environ. Microbiol. 69: 7328-7335.

Fernández, L., Prieto, M. and Guijarro, J. A. (2007) The iron- and temperature-regulated haemolysin YhlA is a virulence factor of *Yersinia ruckeri*. Microbiology. 153: 483-489.

Fernández, L., Secades, P., López, J. R., Márquez, I. and Guijarro, J. A. (2002) Isolation and analysis of a protease gene with an ABC transport system in the fish pathogen *Yersinia ruckeri*: insertional mutagenesis and involvement in virulence. Microbiology. 148: 2233-2243.

Flett, D. E. (1989) O-antigen serogroups of *Yersinia ruckeri*. M.Sc. Thesis, University of Guelph.

Fouz, B., Zarza, C. and Amaro, C. (2006) First description of non-motile *Yersinia ruckeri* serovar I strains causing disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum), cultured in Spain. J. Fish Dis. 29: 339-346.

Frerichs, G. N. (1993) Isolation and identification of fish bacterial pathogens. In: Inglis, V., Roberts, R. J. and Bromage, N. R. (eds.) Bacterial Diseases of Fish. Blackwell Scientific Publications, Oxford, USA. p 270-272.

Frerichs, G. N., Stewart, J. A. and Collins, R. O. (1985) Atypical infection of rainbow trout, *Salmon gairdneri* Richardson, with *Yersinia ruckeri*. J. Fish Dis. 8: 383-387.

Fuhrmann, H., Böhm, K.H., Schlodtfeldt, H.-J. (1984) On the importance of enteric bacteria in the bacteriology of freshwater fish. Bull. Eur. Ass. Fish Pathol. 4: 42-46.

Furones, M. D., Gilpin, M. J., Alderman, D. J. and Munn, C. B. (1990) Virulence of *Yersinia-ruckeri* serotype-I strains is associated with a heat-sensitive factor (*hsf*) in cell-extracts. FEMS Microbiol. Lett. 66: 339-343.

Furushita, M., Shiba, T., Maeda, T., Yahata, M., Kaneoka, A., Takahashi, Y., Torii, K., Hasegawa, T. and Ohta, M. (2003) Similarity of tetracycline resistance genes isolated from fish farm bacteria to hose from clinical isolates. Appl. Environ. Microbiol. 69: 5336-5342.

Gabbianelli, R., Scotti, R., Ammendola, S., Petrarca, P., Nicolini, L. and Battistoni, A. (2011) Role of ZnuABC and ZinT in *Escherichia coli* O157:H7 zinc acquisition and interaction with epithelial cells. BMC Microbiol. 21: 11-36.

Galanos, C., Luderitz, O., Rietschel, E. T., Westphal, O., Brade, H., Brade, L., Freudenberg M., Schade, U., Imoto, M., Yoshimura, H., Kusumoto, S. and Shiba, T. (1985) Synthetic and natural *Escherichia coli* free lipid A express identical endotoxic activities. Eur. J. Biochem. 148: 1-5.

García, J. A., Domínguez, L., Larsen, J. L. and Pedersen, K. (1998) Ribotyping and plasmid profiling of *Yersinia ruckeri*. J. Appl. Microbiol. 85: 949-955.

Gibello, A., Blanco, M. M., Moreno, M. A., Cutuli, M. T., Domenech, A., Dominguez, L. and Fernandez-Garayzabal, J. F. (1999) Development of a PCR assay for detection of *Yersinia ruckeri* in tissues of inoculated and naturally infected trout. Appl. Environ. Microbiol. 65: 346-350.

Gibello, A., Porrero, M. C., Blanco, M. M., Vela, A. I., Liebana, P., Moreno, M. A., Fernandez-Garayabal, J. F. and Dominguez, L. (2004) Analysis of the *gyr*A gene of clinical *Yersinia ruckeri* isolates with reduced susceptibility to quinolones. Appl. Environ. Microbiol. 70: 599-602.

Giorgetti, G., Ceschia, G. and Bovo, G. (1985) First isolation of *Yersinia ruckeri* in farmed rainbow trout in Italy, In*:* Ellis, A. E (ed.). Fish Shellfish Pathology. Academic Press, London. p 161-166.

Glenn, R. A., Taylor, P. W. and Hanson, K. C. (2011) The use of a real-time PCR primer/probe set to observe infectivity of *Yersinia ruckeri* in Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), and steelhead trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis. 34: 783-791.

Guilvout, I., Quilici, M. L., Rabot, S., Lesel, R. and Mazigh, D. (1988). *Bam*HI restriction endonuclease analysis of *Yersinia ruckeri* plasmids and their relatedness to the genus *Yersinia* 42- to 47-megadalton plasmid. Appl. Environ. Microbiol. 54: 2594-2597.

Gunasena, D. K., Komrower, J. and Macintyre, S. (2004) The fish pathogen *Yersinia ruckeri*; possesses a TTS system. In*:* Skurnik, M., Bengoechea, J. A. and Granfors, K. (eds.) The genus Yersinia. Kluwer Academic Publishers.

Guttman, D. S. and Dykhuizen, D. E. (1994) Clonal divergence in *Escherichia coli* as a result of recombination, not mutation. Science 266: 1380-1383.

Hastein, T., Gudding, R. and Evensen, O. (2005) Bacterial vaccines for fish -an update of the current situation worldwide. Progr. Fish Vaccinol. 121: 55-74.

Henryon M., Berg P., Olesen N.J., Kjær T.E., Slierendrecht W.J., Jokumsen A. and Lund I. (2005) Selective breeding provides an approach to increase resistance of rainbow trout (*Onchorhynchus mykiss*) to the diseases, enteric redmouth disease, rainbow trout fry syndrome, and viral haemorrhagic septicaemia. Aquaculture. 250: 621-636.

Heppell, J. and Davis H. L. (2000) Application of DNA vaccine technology to aquaculture. Adv. Drug. Deliv. Rev. 43: 29-43.

Hinshaw, J. M. (1990) Trout Farming-Feeds and feeding methods. Southern Regional Aquaculture Center Publication 223. Stoneville, MS.

Horne, M.T. and Barnes, A. C. (1999). Enteric Redmouth Disease (*Yersinia ruckeri*). In: Woo, P. T.K., and Bruno, D. W. (eds.) Fish Diseases and Disorders Volume 3 Viral, Bacterial and Fungal Infections. CABI Publishing New York, USA. pp. 455-477.

Hudson, K. J., Bliska, J. B. and Bouton, A. H. (2005) Distinct mechanisms of integrin binding by *Yersinia pseudotuberculosis* adhesins determine the phagocytic response of host macrophages. Cell Microbiol. 7: 1474-1489.

Hunter, V. A., Knittel, M. D. and Fryer, J. L. (1980) Stress-induced transmission of *Yersinia-ruckeri* infection from carriers to recipient steelhead trout *Salmo gairdneri* richardson. J. Fish Dis. 3: 467-472.

Hurst, M. R. H., Becher, S. A., Young, S. D., Nelson, T. L. snd Glare, T. R. (2011) *Yersinia entomophaga* sp. nov., isolated from the New Zealand grass grub *Costelytra zealandica*. Int. J. Syst. Evol. Microbiol. 61: 844-849.

Joh, S. J., Kweon, C. H., Kim, M. J., Kang, M. S., Jang, H., Kwon, J. H. (2010) Characterization of *Yersinia ruckeri* isolated from the farm-cultured eel *Anguilla japónica* in Korea. Korean J. Vet. Res. 50: 29-35.

Johns, R. O. (2010) Genetic analysis of strain differences in the fish pathogen *Yersinia ruckeri*. M.Sc. Thesis, University of Guelph.

Kanno, T., Nakai, T. and Muroga, K. (1989) Mode of transmission of vibriosis among ayu *Plecoglossus altivelis*. J. Aquat. Anim. Health 1: 2-6.

Kawula, T. H., Lelivelt, M. J. and Orndorff, P. E. (1996) Using a new inbred fish model and cultured fish tissue cells to study *Aeromonas hydrophila* and *Yersinia ruckeri* pathogenesis. Microbiol. Pathogens. 20: 119-25.

Keeling, S. E., Johnston, C., Wallis, R., Brosnahan, C. L., Gudkovs, N. and McDonald, W. L. (2012) Development and validation of real-time PCR for the detection of *Yersinia ruckeri*. J. Fish Dis. 35: 119-125.

Kim, D. H. and Austin, B. (2008). Characterization of probiotic carnobacteria isolated from rainbow trout (*Oncorhynchus mykiss*) intestine. Lett. Appl. Microbiol. 47: 141-147.

Kollanoor, A., Vasudevan, P., Nair, M. K. M., Hoagland, T. and Venkitanarayanan, K. (2007) Inactivation of bacterial fish pathogens by medium-chain lipid molecules (caprylic acid, monocaprylin and sodium caprylate). Aquacult. Res. 38: 1293-1300.

Kotetishvili, M., Kreger, A., Wauters, G., Morris, J. G., Sulakvelidze, A. and Stine, O. C. (2005) Multilocus sequence typing for studying genetic relationships among *Yersinia* species. J. Clin. Microbiol. 43: 2674-2684.

Ledo, A., Dopazo, C. P., Romalde, J. L., Toranzo, A. E. and Barja, J. L. (1987) Effectiveness of different chemotherapeutic agents for controlling bacterial fish diseases. Bull. Eur. Ass. Fish Pathol. 7: 20-22.

Lehman, J., Sturenberg, F. J. and Mock, D. (1987) The changes in haemogram of rainbow trout (*Salmo gairdneri*, Richardson) to an artificial and natural challenge with *Yersinia ruckeri*. J. Appl. Ichthyol. 3: 174-183.

Lejeune, J. and Rurangirwa, F. (2000) Polymerase chain reaction for definitive identification of *Yersinia ruckeri*. J. Vet. Diagn. Invest. 12: 558-561.

Lemey, P., Rambaut, A., Drummond, A. J. and Suchard, M. A. (2009) Bayesian phylogeography finds its roots. PLoS Comput. Biol. 5: e1000520.

Lesel, R., Lesel, M., Gavini, F. and Vuillaume, A. (1983) Outbreak of enteric redmouth disease in rainbow trout, *Salmo gairdneri* Richardson, in France. J. Fish Dis. 6: 385-387.

Ling, S. H. M., Wang, X. H., Lim, T. M. and Leung, K. Y. (2001) Green fluorescent protein-tagged *Edwardsiella tarda* reveals portal of entry in fish. FEMS Microbiol. Lett.194: 239-243.

Llewellyn, L. (1980) A bacterium with similarities to the redmouth bacterium and *Serratia liquefaciens* (Grimes and Hennerty) causing mortalities in hatchery reared salmonids in Australia. J. Fish Dis. 3: 29-39.

Lucangeli, C., Morabito, S., Caprioli, A., Achene, L., Busani, L., Mazzolini, E., Fabris, A. and Macrì, A. (2000) Molecular fingerprinting of strains of *Yersinia ruckeri* serovar o1 and *Photobacterium damsela* subsp. *piscicida* isolated in Italy. Vet. Microbiol. 76: 273-281.

Madigan, M. T. and Martinko, J. M. (2006) Brock Biology of Microorganisms. 11 ed. Pearson Prentice Hall. Lebanon, USA.

Maiden, M. C. (2006) Multilocus sequence typing of bacteria. Annu. Rev. Microbiol. 60: 561-588.

Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D. A., Feavers, I. M., Achtman, M. and Spratt, B. G. (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. USA 95: 3140-3145.

Mantle, M. and Husar, S. D. (1993) Adhesion of *Yersinia enterocolitica* to purified rabbit and human intestinal mucin. Infect. Immun. 61: 2340-2346.

Marco-Noales, E., Milan, M., Fouz, B., Sanjuan, E. and Amaro, C. (2001) Transmission to eels, portals of entry, and putative reservoirs of *Vibrio vulnificus* serovar E (biotype 2). Appl. Environ. Microbiol. 67: 4717-4725.

Martcheva, M., Bolker, B. M. and Holt, R. D. (2008) Vaccine-induced pathogen strain replacement: What are the mechanisms? J. R. Soc. Interface 5: 3-13.

Maynard, J., Smith, N. H., O'rourke, M. and Spratt, B. G. (1993) How clonal are bacteria?. Proc. Natl. Acad. Sci. USA. 90: 4384-4388.

McArdle, J. F. and Dooley Martin, C. (1985) Isolation of *Yersinia ruckeri* type I (Hagerman strain) from goldfish *Carassius auratus* (L). Bull. Eur. Ass. Fish Pathol. 5: 10-11.

McDaniel, D. W. (1971) Hagerman redmouth. American Fishes U.S. Trout News, 15, 14-28.

Meier, W. (1986) Enteric redmouth disease: outbreak in rainbow trout in Switzerland. Dis. Aqua. Org. 2: 82-86.

Méndez, J. (2012) *Yersinia ruckeri*: genes relacionados con la virulencia y progression del proceso infeccioso. Tesis doctoral, Universidad de Oviedo.

Méndez, J., Fernández, L., Menéndez, A., Reimundo, P., Pérez-Pascual, D., Navais, R. and Guijarro, J. A. (2009) A chromosomally located *traHIJKCLMN* operon encoding a putative type IV secretion system is involved in the virulence of *Yersinia ruckeri*. Appl. Environ. Microbiol. 75: 937-945.

Merhej, V., Adékambi, T., Pagnier, I., Raoult, D. and Drancourt, M. (2008) *Yersinia massiliensis* sp. nov., isolated from fresh water. Int. J. Syst. Evol. Microbiol. 58: 779-784.

Michel, C., Faivre, B. and de Kinkelin, P. (1986) A clinical case of enteric redmouth in minnows (*Pimephales promelas*) imported in Europe as baitfish. Bull. Eur. Ass. Fish Pathol. 6: 97-99.

Mitchum, D. L. (1981) Concurrent infections: ERM and furunculosis found in esmerald shiners. Am. Fish. Soc. Fish Health Newsl. 9: 2.

Moodley, Y., Linz, B., Yamaoka, Y., Windsor, H. M., Breurec, S., Wu, J.-Y., Maady, A., Bernhöft, S., Thiberge, J.-M., Phuanukoonnon, S., Jobb, G., Siba, P., Graham, D. Y., Marshall, B. J. and Achtman, M. (2009) The

peopling of the Pacific from a bacterial perspective. Science 323: 527-530.

Morelli, G., Song, Y. J., Mazzoni, C. J., Eppinger, M., Roumagnac, P., Wagner, D. M., Feldkamp, M., Kusecek, B., Vogler, A. J., Li, Y., Cui, Y., Thomson, N. R., Jombart, T., Leblois, R., Lichtner, P., Rahalison, L., Petersen, J. M., Balloux, F., Keim, P., Wirth, T., Ravel, J., Yang, R., Carniel, E. and Achtman, M. (2010) *Yersinia pestis* genome sequencing identifies patterns of global phylogenetic diversity. Nat. Genet. 42: 1140-1143.

Morrison, D. C. and Ryan, J. L. (1987) Endotoxins and disease mechanisms. Ann. Rev. Med. 38: 417-432.

Murros-Kontiainen, A., Fredriksson-Ahomaa, M., Korkeala, H., Johanson, P., Rahkila, R. and Björkroth, J. (2011) *Yersinia nurmii* sp. nov. Int. J. Syst. Evol. Microbiol. 61: 2368-2372.

Nakai, T. and Park, S. C. (2002) Bacteriophage therapy of infectious diseases in aquaculture. Res. Microbiol. 153: 13-18.

Neilands, J. B. (1995) Siderophores: structure and function of microbial iron transport compounds. J. Biol. Chem. 270: 26723-26726.

Noga, E. J., Levine, J. F., Townsend, K., Bullis, R. A., Carlson, C. P. and Corbett, W. T. (1988) Kidney biopsy: a nonlethal method for diagnosing *Yersinia ruckeri* infection (enteric redmouth disease) in rainbow trout *Salmo gairdneri*. Am. J. Vet. Res. 49: 363-365.

O'Leary, P. J. (1977) Enteric redmouth bacterium of salmonids: a biochemical and serological comparison of selected isolates. M.Sc. Thesis, Oregon State University, Corvallis.

O'Leary, P. J., Rohovec, J. S. and Fryer, J. L. (1979). A further characterization

of *Yersinia ruckeri* (enberic redmouth bacterium). Fish Pathol. 14: 71-78.

Olsson, J. C., Jöborn, A., Westerdahl, A., Blomberg, L., Kjelleberg, S. An

Conway, P. L. (1996) Is the turbot, *Scophthalmus maximus* (L.), intestine a portal of entry for the fish pathogen *Vibrio anguillarum*? J. Fish Dis. 19: 225-234.

Ostrowski, C. (2012) In vivo bioluminescent imaging in fish and intraspecies typing of *Yersinia ruckeri*. PhD Thesis. University of Guelph.

Palm, R., Landolt, M. L. and Busch, R. A. (1998) Route of vaccine administration: effects on the specific humoral response in rainbow trout *Oncorhynchus mykiss*. Dis. Aquat. Org. 33: 157-166.

Petrarca, P., Ammendola, S., Pasquali, P. and Battistoni, A. (2010) The Zur-regulated ZinT protein is an auxiliary component of the high-affinity ZnuABC zinc transporter that facilitates metal recruitment during severe zinc shortage. J. Bacteriol. 192: 1553-1564

Pfeiffer, A. (1889) Ueber die bäcillare Pseudotuberkulose bei Nagethieren, Thieme, Lipzig.

Poole, K., Schiebel, E. and Braun, V. (1988) Molecular characterization of the hemolysin determinant of *Serratia marcescens*. J. Bacteriol. 170: 3177-3188.

Post, G. (1987) Ulcer disease. In: Text Book of Fish Health. TFH Publication Inc., USA. Vol. 1043: 69-70.

Pulkkinen, K., Suomalainen, L.-R., Read, A. F., Ebert, D., Rintamäki, P. and Valtonen, E. T. (2010) Intensive fish farming and the evolution of pathogen virulence: the case of columnaris disease in Finland. Proc. R. Soc. B: Biol. Sci. 277: 593-600.

Quentel, C. and Aldrin, J. F. (1986) Blood changes in catheterised rainbow trout (*Salmo gairdneri*) intraperitoneally inoculated with *Yersinia ruckeri*. Aquaculture. 53: 169-185.

Raida, M. K. and Buchmann, K. (2008) Development of adaptive immunity in rainbow trout, *Oncorhynchus mykiss* (Walbaum) surviving an infection with *Yersinia ruckeri*. Fish Shellfish Immunol. 25: 533-541.

Raida, M. K. and Buchmann, K. (2009) Innate immune response in rainbow trout (*Oncorhynchus mykiss*) against primary and secondary infections with *Yersinia ruckeri* O1. Dev. Comp. Immunol. 33: 35-45.

Raida, M. K., Larsen, J. L., Nielsen, M. E. and Buchmann, K. (2003) Enhanced resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Yersinia ruckeri* challenge following oral administration of *Bacillus subtilis* and *B. licheniformis* (BioPlus2B). J. Fish Dis. 26: 495-498.

Reeves, P. (1995) Role of O-antigen variation in the immune response. Trend. Microbiol. 3: 381-386.

Rigos, G. and Stevenson, R. (2001) The effect of antibiotic treatment on the establishment of persistent infection with *Yersinia ruckeri* Serovar II in rainbow trout *Oncorhynchus mykiss* (Walbaum). Aquac. Int. 9: 247-253.

Rintamäki, P., Valtonen, E. T. and Frerichs, G. N. (1986) Occurrence of *Yersinia ruckeri* infection in farmed whitefish, *Coregonus peled* Gmelin and *Coregonus muksun* Pallas, and Atlantic salmon, *Salmo salar* L., in northern Finland. J. Fish Dis. 9: 137-140.

Roberts, M. S. (1983) A report of an epizootic in hatchery reared rainbow-trout, *Salmo gairdneri* Richardson, at an English trout farm, caused by *Yersinia ruckeri*. J. Fish Dis. 6: 551-552.

Roberts, R. J. (ed.) (2001) Fish Pathology, 3rd ed. Philadelphia: W. B.

Saunders. 472 pp.

Rodgers, C. J. (1992) Development of a selective-differential medium for the isolation of *Yersinia ruckeri* and its application in epidemiological studies. J. Fish Dis. 15: 243-254.

Rodgers, C. J. (2001). Resistance of *Yersinia ruckeri* to antimicrobial agents *in vitro*. Aquaculture. 196: 325-345.

Rodgers, C. J. and Austin, B. (1983) Oxolinic acid for control of enteric redmouth disease in rainbow trout. Vet. Rec. 112: 83.

Romalde, J. L. (1992) *Yersinia ruckeri*: Estudio epidemiológico y del mecanismo de virulencia. Tesis doctoral. Universidad de Santiago de Compostela.

Romalde, J. L. and Toranzo, A. E. (1991) Evaluation of the API-20E system for the routine diagnosis of the enteric redmouth disease. Bull. Eur. Ass. Fish Pathol. 11: 147-149.

Romalde, J. L. and Toranzo, A. E. (1993) Pathological activities of *Yersinia ruckeri*, the enteric redmouth (ERM) bacterium. FEMS Microbiol. Lett. 112: 291-299.

Romalde, J. L., Barja, J. L., Magariños, B., and Toranzo, A. E. (1994) Starvation-survival processes of the fish bacterial pathogen *Yersinia ruckeri*. Syst. Appl. Microbiol. 17: 161-168.

Romalde, J. L., Conchas, R. F. and Toranzo, A. E. (1991) Evidence that *Yersinia ruckeri* possesses a high affinity iron uptake system. FEMS Microbiol. Lett. 64: 121-125.

Romalde, J. L., Lemos, M. L., Conchas, R. F., Bandín, I. and Toranzo, A. E. (1990) Adhesive properties and other virulence factors in *Yersinia*

ruckeri. In: Cheng, T.C. and Perkins, F.O. (eds.). Pathology in Marine Science. Academic Press, New York. p. 123-139.

Romalde, J. L., Margariños, B., Barja, J. L., and Toranzo, A. E. (1993) Antigenic and molecular characterization of *Yersinia ruckeri*. Proposal for a new intraspecies classification. Syst. Appl. Microbiol. 16: 411-419.

Romalde J. L., Planas, E., Sotelo J. M. and Toranzo A. E. (2003) First description of *Yersinia ruckeri* serotype O2 in Spain. Bull. Eur. Assoc. Fish Pathol. 23: 135-138.

Ross, A. J., Rucker, R. R. and Ewing, W. H. (1966) Description of a bacterium associated with redmouth disease of rainbow trout (*Salmo gairdneri*). Can. J. Microbiol. 12: 763-770.

Rucker, R. R. (1966) Redmouth disease of rainbow trout (*Salmo gairdneri*). Bull. l'Offic. Int. Epizoot. 65: 825-830.

Ryckaert, J., Bossier, P., D'herde, K., Diez-fraile, A., Sorgeloos, P., Haesebrouck, F. and Pasmans, F. (2010) Persistence of *Yersinia ruckeri* in trout macrophages. Fish Shellfish Immunol. 29: 648-655.

Sabri, M., Houle, S. and Dozois, C. (2009) Roles of the extraintestinal pathogenic *Escherichia coli* ZnuACB and ZupT zinc transporters during urinary tract infection. Infect. Immun. 77: 1155-1164.

Saleh, M., Soliman, H. and El-Matbouli, M. (2008) Loop-mediated isothermal amplification as an emerging technology for detection of *Yersinia ruckeri* the causative agent of enteric red mouth disease in fish. BMC Vet. Res. 4: 31.

Santos, Y., Bandín, I., Núñez, S., Gravningen, K. and Toranzo, A. E. (1991) Protection of turbot, *Scophthalmus maximus* (L.), and rainbow trout, *Oncorhynchus mykiss* (Richardson), against vibriosis using two different

vaccines. J. Fish Dis. 14: 407-411.

Sauter, R. W., Williams, C., Celnik, B. and Meyer, E. A. (1985) Etiology of early lifestage diseases, Department of Microbiology and Immunology, Oregon Health Sciences University, Final Report.

Schleifstein, J. and Coleman, M. B. (1943). *Bacterium enteroliticum*, N. Y. State Dep. Health Div. Lab. Res. Annu. Report. 56.

Schill, W. B., Phelps, S. R. and Pyle, S. W. (1984) Multilocus electrophoretic assessment of the genetic-structure and diversity of *Yersinia-ruckeri*. Appl. Environ. Microbiol. 48: 975-979.

Schmidt, A. S., Bruun, M. S., Dalsgaard I., Pedersen, K. and Larsen J. L. (2000) Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. Appl. Environ. Microbiol. 66: 4908-4915.

Schroers, R., Griesinger, F., Trumper, L., Haase, D., Kulle, B., Klein-Hitpass, L., Sellmann, L., Dührsen, U. and Dürig, J. (2005) Combined analysis of ZAP-70 and CD38 expression as a predictor of disease progression in B-cell chronic lymphocytic leukemia. Leukemia. 19: 750-758.

Secades, P. and Guijarro, J. A. (1999) Purification and characterization of an extracellular protease from the fish pathogen *Yersinia ruckeri* and effect of culture conditions on production. Appl. Environ. Microbiol. 65: 3969-3975.

Siwicki, A. K., Morand, M., Fuller, J. C. Jr., Nissen, S., Goryczko, K., Ostaszewski, P., Kazun, K. and Glompski, E. (2003) Influence of feeding the leucine metabolate ß-hydroxy ß-methyl butyrate (HMB) on the non-specific cellular and humoral defence mechanisms of rainbow trout (*Oncorhynchus mykiss*). J. Appl. Ichthyol. 19: 44-48.

Smith, A. M., Goldring, O. L. and Dear, G. (1987) The production and methods of use of polyclonal antisera to the pathogenic organisms *Aeromonas salmonicida*, *Yersinia ruckeri* and *Renibacterium salmoninarum*. J. Fish Biol. 31: 225-226.

Smith, K. D., Andersen-Nissen, E., Hayashi, F., Strobe, K., Bergman, M. A., Barrett, S. L., Cookson, B. T. and Aderem, A. (2003) Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat. Immunol. 4: 1247-1253.

Sousa, J. A., Margariños, B., Eiras, J. C., Toranzo, A. E. and Romalde J. L. (2001) Molecular characterization of Portuguese strains of *Yersinia ruckeri* isolated from fish culture systems. J. Fish Dis. 24: 151-159.

Spanggaard, B., Huber, I., Nielsen, J., Nielsen, T. and Gram, L. (2000) Proliferation and location of *Vibrio anguillarum* during infection of rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis. 23: 423-427.

Sparboe, O., Koren, C., Hastein, T., Poppe, T. and Stenwig, H. (1986) The first isolation of *Yersinia ruckeri* from farmed Norwegian salmon. Bull. Eur. Ass. Fish Pathol. 6: 41-42.

Spencer, E., Garrett, C., Dos Santos, L. and Jahnke, M. L. (1997) Public, animal and environmental health implications of aquaculture. Emerg. Infect. Dis. 3: 453-457.

Sprague, L. D. and Neubauer, H. (2005) *Yersinia aleksiciae* sp. nov. Int. J. Syst. Evol. Microbiol. 55: 831-835.

Sprague, L. D., Scholz, H. C., Amann, S., Busse, H.-J. and Neubauer, H. (2008) *Yersinia similis* sp. nov. Int. J. Syst. Evol. Microbiol. 58: 952-958.

Spratt, B. G., Hanage, W., Li, B., Aanensen, D. and Feil, E. (2004) Displaying

the relatedness among isolates of bacterial species the eBURST approach. FEMS Microbiol. Lett. 241: 129-134.

Stave, J. W., Cook, T. M. and Robertson, B. S. (1987) Chemiluminescent responses of striped bass, *Morone saxatilis* (Walbaum), phagocyte to strains of *Yersinia ruckeri*. J. Fish Dis. 10: 1-10.

Stevenson R. M. W. (1997) Immunization with bacterial antigens: yersiniosis. Dev. Biol. Standard. 90: 117-124.

Stevenson, R. M. W. and Airdrie, D. W. (1984a) Isolation of *Yersinia ruckeri* bacteriophages. Appl. Environ. Microbiol. 47: 1201-1205.

Stevenson, R. M. W. and Airdrie, D. W. (1984b) Serological variation among *Yersinia-ruckeri* strains. J. Fish Dis. 7: 247-254.

Stevenson, R. M. W. and Daly, J. G. (1982) Biochemical and serological characteristics of Ontario isolates of *Yersinia-ruckeri*. Can. J. Fisheries Aquat. Sci. 39: 870-876.

Stevenson, R., Flett, D. and Raymon, B. T. (1993) Enteric Redmouth (ERM) and other enterobacterial infections. In: Inglis, V., Roberts, R.I. and Bromage, N.R. (eds.). Bacterial Diseases of Fish. Blackwell Scientific Publications. Edinburgh. pp. 80-106.

Ström-Bestor, M., Mustamäki, N., Heinikainen, S., Hirvelä-koski, V., Verner-Jeffreys, D. and Wiklund, T. (2010) Introduction of *Yersinia ruckeri* biotype 2 into Finnish fish farms. Aquaculture. 308: 1-5.

Sullivan, J. (1981) Enteric redmouth. In: Dieretich, R. A. (eds.). Alaskan Wildlife Disease. University of Alaska, Fairbanks, Workshop. p 406-410.

Svendsen, Y. S. and Bøgwald, J. (1997) Influence of artificial wound and non-intac mucus layer on mortality of Atlantic salmon (*Salmo solar* L.)

following a bath challenge with *Vibrio anguillarum* and *Aeromonas salmonicida*. Fish Shellfish Immunol. 7: 317-325.

Swaminathan B. Barrett TJ. Hunter SB, Tauxe, R. V. and CDC PulseNet Task Force. (2001) PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerg. Infect. Dis. 7: 382-389.

Tacon, A. G. J., Metian, M., Turchini, G. M. and De Silva, S. S. (2010) Responsible aquaculture and trophic level implications to global fish supply. Rev. Fisheries Sci. 18: 94-105.

Tankouo-Sandjong, B., Sessitsch, A., Liebana, E., Kornschober, C., Allerberger, F., Hächler, H. and Bodrossy, L. (2007) MLST-V, multilocus sequence typing based on virulence genes, for molecular typing of *Salmonella enterica* subsp. *enterica* serovars. J. Microbiol. Methods. 69: 23-36.

Tatner, M. F. and Horne, M. T. (1985) The effects of vaccine dilution, length of immersion time, and booster vaccinations on the protection levels induced by direct immersion vaccination of brown trout, *Salmo trutta*, with *Yersinia ruckeri* (ERM) vaccine. Aquaculture. 46: 11-18.

Temprano, A., Riano, J., Yugueros, J., Gonzalez, P., Castro, L., Villena, A., Luengo, J. M. and Naharro, G. (2005) Potential use of a *Yersinia ruckeri* O1 auxotrophic *Aroa* mutant as a live attenuated vaccine. J. Fish Dis. 28: 419-427.

Temprano, A., Yugueros, J., Hernanz, C., Sánchez, M., Berzal, B., Luengo, J. M. and Naharro, G. (2001) Rapid identification of *Yersinia ruckeri* by PCR amplification of *yru*I-*yru*R quorum sensing. J. Fish. Dis. 24*:* 253-261.

Thorsen, B. K., Enger, O., Norland, S. and Hoff, K. A. (1992) Long-term starvation survival of *Yersinia ruckeri* at different salinities studied by microscopical and flow cytometric methods. Appl. Environ. Microbiol. 58: 1624-1628.

Tinsley, J. W., Lyndon, A. R. and Austin, B. (2011) Antigenic and cross-protection studies of biotype 1 and biotype 2 isolates of *Yersinia ruckeri* in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Appl. Microbiol. 111: 8-16.

Tobback, E., Decostere, A., Hermans, K., Haesebrouck, F. and Chiers, K. (2007) *Yersinia ruckeri* infections in salmonid fish. J. Fish Dis. 30: 257-268.

Toledo, M. S., Troncoso, M., Portell, D. P., Figueroa, G. (1993) Outbreak due to *Yersinia ruckeri* in cultured salmonids. Ann. Microbiol. 1:59-62.

Topic, N., Coz-rakovac, R. and Strunjak-Perovic, I. (2007) Commercial phenotypic tests (API 20E) in diagnosis of fish bacteria: a review. Veterinari Medicina 52:49-53.

Toranzo, A. E., Baya, A. M., Roberson, B. S., Barja, J. L., Grimes, D. J. and Hetrick, F. M. (1987) Specificity of slide agglutination test for detecting bacterial fish pathogens. Aquaculture 61: 81-97.

Ursing, J., Brenner, D. J., Bercovier, H., Fanning, G. R., Steigerwalt, A. G., Brault, J. and Mollaret, H. H. (1980) *Yersinia frederiksenii*: a new species of *Enterobacteriaceae* composed of rhamnose-positive strains (formerly called atypical *Yersinia enterocolitica* or *Yersinia enterocolitica*-like). Curr. Microbiol. 4: 213-217.

Valtonen, E. T, Rintamäki, P. and Koskivaara, M. (1992) Occurence and pathogenicity of *Yersinia ruckeri* at fish farms in northern and central

Finland: Do wild fish serve as a source of infection? J. Fish Dis. 15:163-171.

Van Loghem J. J. (1944) The classification of the plague bacillus. Antonie van Leewenhoek J. Serol. Microbiol. 10: 15-16.

Vendrell D., Balcázar, J., Calvo, A., de Blas, I., Ruiz-Zarzuela, I., Gironés, O. and Múzquiz, J. (2009) Quantitative analysis of bacterial adhesion to fish tissue. Coll. Surf. B: Biointerfaces. 71: 331-333.

Venecia, K. and Young, G. M. (2005) Environmental regulation and role in pathogenesis of the Ysa type III secretion system of *Yersinia enterocolitica* Biovar 1B. Infect. Immun. 73: 5961-5977.

Vivas, J., Carracedo, B., Riaño, J., Razquin, B. E., López-Fierro, P., Acosta, F., Naharro, G. and Villena, A. J. (2004) Behavior of an *Aeromonas hydrophila* aroA live vaccine in water microcosms. Appl. Environ. Microbiol. 70: 2702-2708.

Vuillaume, A., Brun, R., Chene, P., Sochon, E. and Lessel, R. (1987) First isolation of *Yersinia ruckeri* from sturgeon, *Acipenser baeri* Brandt, in south west of France. Bull. Eur. Ass. Fish Pathol. 7: 18-19.

Wagner, U., Gudmundsdóttir, B. K. and Drössler, K. (1999) Monoclonal antibodies against AsaP1, a major exotoxin of the fish pathogen *Aeromonas salmonicida* subsp. *achromogenes*, and their application in ELISA. J. Appl. Microbiol. 87: 620-629.

Wauters, G., Janssens, M., Steigerwalt, A. G. and Brenner, D. J. (1988) *Yersinia mollaretii* sp. nov. and *Yersinia bercovieri* sp. nov., formerly called *Yersinia enterocolitica* biogroups 3A and 3B. Int. J. Syst. Bacteriol. 38: 424-429.

Welch, J. J. and Bromham, L. (2005) Molecular dating when rates vary. Trends

Ecol. Evol. 20: 320-327.

Welch, T. J. and Wiens, G. D. (2005) Construction of a virulent, green fluorescent protein tagged *Yersinia ruckeri* and detection in trout tissues after intraperitoneal and immersion challenge. Dis. Aquat. Org. 67: 267-272.

Welch, T. J., Verner-Jeffreys, D. W., Dalsgaard, I., Wiklund, T., Evenhuis, J. P., García Cabrera, J. A., Hinshaw, J. M., Drennan. J. D. and LaPatra, S. E. (2011) Independent emergence of *Yersinia ruckeri* biotype 2 in the United States and Europe. Appl. Environ. Microbiol. 77: 3493-3499.

Wheeler, R. W., Davies, R. L., Dalsgaard, I., Garcia, J., Welch, T. J., Wagley, S., Bateman, K. S. and Verner-Jeffreys, D. W. (2009) *Yersinia ruckeri* biotype 2 isolates from mainland Europe and the UK likely represent different clonal groups. Dis. Aquat. Org. 84: 25-33.

Willumsen, B. (1989) Birds and wild fish as potential vectors of *Yersinia ruckeri*. J. Fish Dis. 12: 275 - 277.

Wobeser, G. (1973) An outbreak of redmouth disease in rainbow trout (*Salmo gairdner*i) in Saskatchewan. J. Fisheries Res. Board Can. 30: 571-575.

Young, G. M., Smith, M. J., Minnich, S. A. and Miller, V. L. (1999) The *Yersinia enterocolitica* motility master regulatory operon, *flh*DC, is required for flagellin production, swimming motility, and swarming motility. J. Bacteriol. 181: 2823-2833.

Zamze, S. E. and Moxon, E. R. (1987) Composition of the lipopolysaccharide from different capsular serotype strains of *Haemophilus influenzae*. J. Gen. Microbiol. 133: 1443-1451.

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